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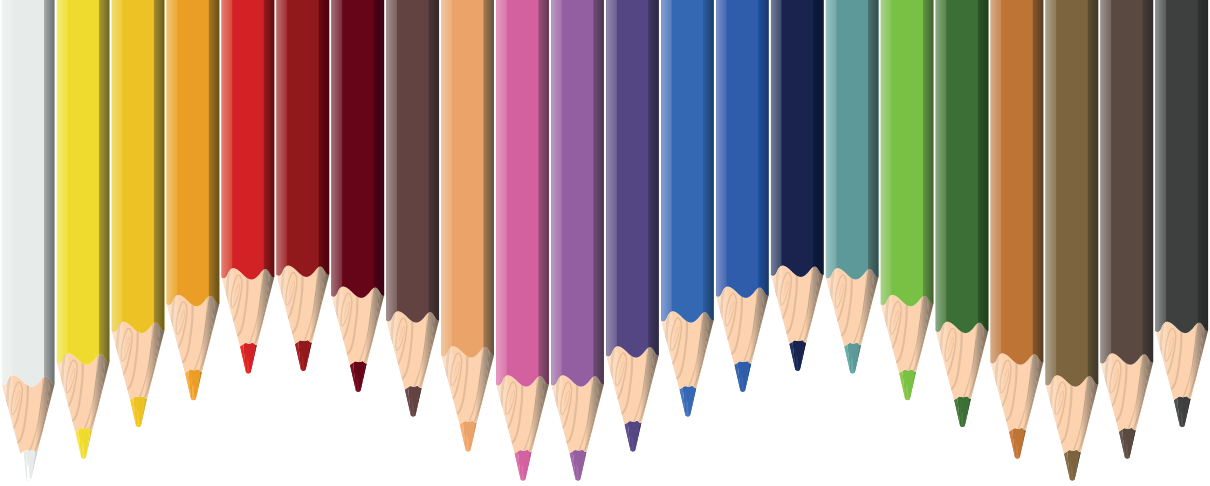

Chapter 1

SIGNIFICANCE OF KINETİC STUDIES ON LACTOPEROXIDASE ENZYME

Hande USANMAZ[1]

1 This study is derived from a section of the doctoral thesis titled "Purification of lactoperoxidase enzyme from bovine and sheep milk by affinity chromatography technique" by the author Hande Usanmaz (YÖK Thesis No: 361144, Atatürk University; Institute of Natural Sciences; Department of Chemistry, 2014).

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Enzymes, the catalysts of biological systems, are protein molecules that facilitate chemical transformations. A significant portion of the human genome encodes information related to enzymes (Lehninger 2005). The primary functions of enzymes include catalytic power and specificity. The active region where catalysis takes place in enzymes is referred to as the active site. The active site comprises units with the ability to bind and break bonds, including the enzyme's substrate and, if present, its cofactor. Enzymes with catalytic properties reduce the activation energy of a reaction, thereby significantly increasing the reaction rate (Berg et al. 2014).

The reaction catalyzed by enzymes is millions of times faster than the corresponding non-catalyzed reaction. Enzymes are highly specific to the reactions they catalyze and the substrates they use during the reaction. These protein molecules undergo no changes after catalyzing reactions, do not deplete, and do not alter the equilibrium of the reaction. Utilizing all forces in the molecules, enzymes bring substrates together in the most suitable way, facilitating the formation and breaking of chemical bonds (Keha and Küfrevioğlu 2012).

Many enzymes require cofactors for their activities, which can be divided into two subgroups: metals and organic molecules called coenzymes. Coenzymes, which are of vitamin origin, can tightly or loosely bind to enzymes. When a coenzyme tightly binds to an enzyme, it is referred to as a prosthetic group. Enzymes are responsible for the conversion of energy from one form to another. For example, photosynthesis converts light energy into chemical bond energy. In the process of respiration occurring in the mitochondria, small molecules obtained from food are transformed by enzymes into a form of free energy that the cell can use (Bingöl 1983, Sigman and Boyer 1990).

The activities of many enzymes can be inhibited by the binding of specific molecules and ions. Inhibition of enzymes in biological systems serves as a control mechanism. Additionally, many drugs can inhibit enzymes, and enzyme inhibition can be reversible or irreversible. Irreversible inhibitors tightly bind to the enzyme and are challenging to dissociate from it. Some drugs act as irreversible enzyme inhibitors. In reversible inhibition, the inhibitor can quickly dissociate from the enzyme and is classified into competitive and non-competitive inhibition (Berg et al. 2014). In competitive inhibition, the enzyme binds to the substrate, forming the enzyme-substrate (ES) complex or the enzyme-inhibitor (EI) complex, and the enzyme-substrate-inhibitor (ESI) complex does not form. In non-competitive inhibition, the inhibitor only binds to the enzyme-substrate complex. Increasing the substrate concentration does not reduce inhibition in this type of inhibition (Gilbert 1992).

In living cells, protection against free radicals and the toxic effects of

oxygen metabolism is necessary for the regulation of metabolic reactions. Antioxidant enzymes and molecules, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, proteases, phospholipases, and peroxidases, are effective in this protective system. Antioxidant enzymes and molecules inhibit lipid peroxidation and other free radical-mediated reactions in aerobic cells, protecting cells against oxidative stress. Antioxidants play a crucial role in preventing the progression of many chronic diseases, such as lipid peroxidation (Gülçin et al. 2010a).

Free radicals are substances that have one or more unpaired electrons in their outer orbitals. Active radicals can oxidize biomolecules, attacking them and potentially damaging biological membrane structures. Molecular oxygen is an ideal molecule for receiving the final electron in the electron transport chain due to its high affinity for electrons, providing a significant thermodynamic driving force. Harmful compounds can be formed during this reduction, and one of them is superoxide, while the other is peroxide. Both compounds formed are potentially destructive to cells. Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$) produced from them are collectively referred to as reactive oxygen species (ROS). ROS can be of metabolic origin, such as in the electron transport system, some enzymatic reactions, and oxidation reactions, or externally induced, such as by UV radiation, radiation, drug side effects, smoking, diet, carcinogenic substances, etc. (Aksoy 2002, Gülçin 2012).

Although cytochrome C oxidase, the enzyme of the electron transport chain that reduces oxygen, and other proteins are successful in not leaving reactive intermediate products, small amounts of superoxide and peroxide are inevitably produced. Superoxide dismutase plays a crucial role as a superoxide collector in the conversion of superoxide to hydrogen peroxide and molecular oxygen. In eukaryotes, this enzyme is localized in the mitochondria and cytoplasm. In mitochondria, this enzyme is bound to copper and zinc. Superoxide dismutase and hydrogen peroxide produced by different processes are captured by the catalase enzyme and converted to molecular oxygen and water. Both enzymes are highly effective. Glutathione peroxidase also plays a significant role in capturing hydrogen peroxide (Doumonted and Rousset 1983).

In addition, vitamin E and vitamin C are effective against oxidative damage, and due to their lipophilic nature, vitamin E is highly effective in protecting cell membranes from peroxidation. Despite the known dangers of ROS, recent studies have shown that these molecules are important components of signal transduction pathways. The contradictory behavior of ROS serves as an example of the complexity of metabolism, demonstrating that potentially hazardous substances can be utilized in beneficial reactions

(Miller 1996).

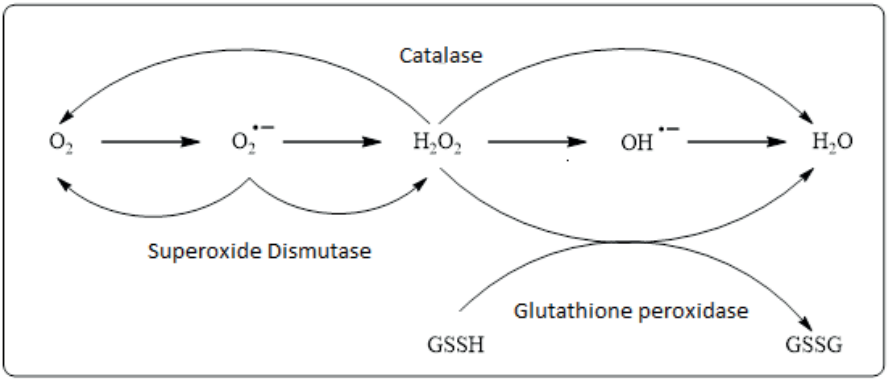


Figure 1.1. *Formation of Free Radicals and Reactive Species*

The characteristics of free radicals and reactive species formation are summarized in Figure 1.1. During metabolism, superoxide dismutase, catalase, and glutathione peroxidase enzymes appear to be highly effective in scavenging radicals (Gökpınar et al., 2006). The hydroxyl radical (OH·-), the neutral form of hydroxide ions, is known as the most reactive species with a half-life of approximately 10^-9 s (Gülçin, 2012). The hydroxyl radical (OH·-) is formed by the reduction of H_2O_2 (Fantal, 1996; Halliwell and Gutteridge, 1989; Nordberg and Arner, 2001). Highly reactive radicals like hydroxyl and hydroperoxyl (HOO·) can initiate lipid peroxidation, leading to protein damage, enzyme inhibition, and denaturation (Gülçin, 2008; Stadtman, 1992; Lenaz, 2001; Elmastas et al., 2006).

Superoxide anion radical (O_2·-) is the most abundant and easily formed oxygen-centered radical during metabolism (Halliwell, 1989). Superoxide anion radical (O_2·-) is converted to hydrogen peroxide through a reaction catalyzed by superoxide dismutase enzyme (Cadenas and Packer, 1996). Hydrogen peroxide, easily formed, disrupts the lipid structure in membrane metabolism and is utilized in peroxidation reactions by many plant peroxidases (Öztürk Sarıkaya, 2009; Adam and Yiğitoğlu, 2012).

1.1. **Peroxidases (POD: H_2O_2-Oxidoreductase E.C.1.11.1.7)**

Reactive oxygen species formed during metabolism are converted into harmless molecules through enzymes (Davies, 1995; Champe et al., 2007). Peroxidases exhibit antioxidant properties by catalyzing the oxidation of organic and inorganic substrates using hydrogen peroxide as an electron acceptor (Hussain et al., 1995). They catalyze the dehydrogenation of various aromatic components such as phenols, hydroquinones, and hydroquinoid amines (only benzidine derivatives). Examples include 2-cresol,

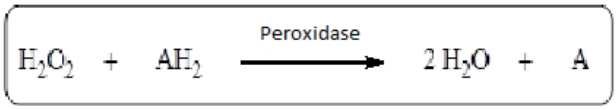
2-toluidine, guaiacol, pyrogallol, homovanillic acid, hydroquinone, 1,2 and 1,4-phenylenediamine, leukomalachite green, 2,6-dichlorophenolindophenol, 4,4-diaminodiphenylamine, propionyl promazine, benzidine, o-toluidine, di-o-anisidine, and some azo dye derivatives (Pütter and Becker, 1987; Van Huystee, 1987).

Peroxidases are commonly found in prokaryotes, eukaryotes, and photosynthetic cells. They are present in various plant species, legumes, high plants such as tobacco, yeasts, fungi, and bacteria. Different peroxidase isoenzymes have been characterized from various plant species. Peroxidase (POD) has numerous isoenzymes with different biochemical properties such as specific activity, substrate affinity, cofactors, sensitivity to inhibitors, and optimum pH (Fric, 1976; Van Huystee, 1987). Plant peroxidases play a role in various functions, including binding of cell wall proteins, germination, defense mechanisms, lignin biosynthesis, hormonal activity, and oxidative stress (Bartonek-Roxa et al., 1991; Morohashi, 2002; Duarte et al., 2000; Wakamatsu and Takahama, 1993; Hiraga et al., 2001).

In mammals, peroxidase enzymes are localized in various tissues and organs, such as lactoperoxidase in milk and salivary glands, myeloperoxidase in leukocytes, thrombocytes, liver, and spleen, glutathione peroxidase in the uterus, lung walls, cytoplasm, and mitochondria. Peroxidase synthesis initially results in a non-functional protein. Functional holoenzyme is formed by combining the heme group with the apoprotein. Peroxidases have various prosthetic groups, with the first being protoheme, which is loosely bound to the apoprotein, unlike many hemoproteins. In reactions catalyzed by peroxidases, H_2O_2 is reduced, causing potential damage to electron acceptors such as ascorbate, quinones, and cytochrome C (Doumonted and Rousset, 1983). The removal of H_2O_2, which exhibits oxidative properties, is essential. This task is carried out by important antioxidant enzymes in cells, namely catalase and peroxidase (Haliwell, 1984; Robert et al., 1993). Antioxidant enzymes also prevent biological oxidative damage by scavenging free oxygen radicals (Harris, 1992).

The second prosthetic group on the peptide chain is glucoseid side chains. Most peroxidase isoenzymes contain carbohydrates, representing about 15-17% of their molecular weight (Van Huystee, 1987). In addition to the heme group and glucoseidic groups, calcium (Ca2+) ions are also required for peroxidase release and structural integrity. Various researchers have reported that horseradish peroxidase has a molecular weight of 31-33.8 kD and contains a glucoseid side chain in addition to the heme group (Van Huystee, 1987).

The reactions catalyzed by peroxidases are generally illustrated as follows (Van Huystee, 1987).



Peroxidases find extensive use in the study of metabolic reactions, enzyme functions, and protein structures due to the ease with which their activities can be measured using chromogenic methods and their resistance to high temperatures (Hiraga et al., 2001). Peroxidases, especially lactoperoxidase, are commonly preferred in clinical diagnosis, microanalytical applications, as well as in the pharmaceutical and food industries (Kwak et al., 1996).

1.2. Lactoperoxidase (LPO)

LPO is a member of the peroxidase family, functioning as an oxidoreductase found in milk, saliva, and tears. It plays a crucial role in protecting the intestinal systems and mammary glands of newborns against pathogenic microorganisms. LPO is a normal component of both bovine and human milk, exhibiting similar chemical and immunological properties (Kumar and Bhatla, 1995). Being a basic protein, LPO has an isoelectric pH value of 9.2 (Kussendrager and van Hooijdonk, 2000).

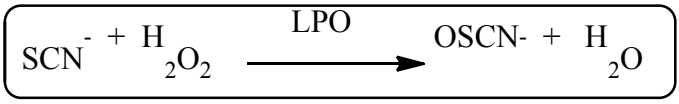
Comprising 612 amino acid residues, a single heme prosthetic group, and four or five carbohydrate chains, LPO is a single polypeptide chain with an approximate molecular weight of 85 kDa, constituting around 10% of the total mass (Paul et al., 1985; Elagamy et al., 1992; Reiter and HaÈrnulv, 1984; Sisecioglu et al., 2010). The catalytic center contains a heme group of protoporphyrin IX, covalently bound to the polypeptide chain via a disulfide bridge (Thanabal and La Mar, 1989). The iron composition in LPO is 0.07% (Booth et al., 1989).

Derived from mammalian milks, LPO enzyme is highly significant in suppressing bacterial growth and supporting bacterial inhibition (Jacob et al., 1998). The inhibitory effect of bovine LPO on bacterial growth is attributed to the peroxidase system containing H_2O_2 and thiocyanate (Jacob et al., 1998). This system's antimicrobial effect occurs naturally in milk (Haddain et al., 1996). LPO exhibits a bacteriostatic effect on both gram-positive and gram-negative bacteria. Antibacterial studies on LPO have revealed significant inhibition in pathogenic bacteria by the LPO-thiocyanate and peroxide system. LPO has diverse applications, serving as a preservative in both food and pharmaceutical industries, and is used in the dairy industry for preserving milk during transportation (Barrett et al., 1999).

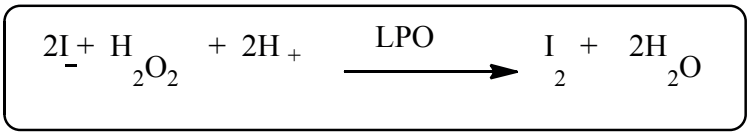
LPO utilizes H_2O_2 to generate potent oxidizing and bactericidal substances by oxidizing halides and pseudohalides such as thiocyanate. LPO

catalyzes the antibacterial oxidation of endogenous hypothiocyanate and is a redox enzyme with antibacterial properties found in biological fluids such as milk and saliva (Cals et al., 1991; Jacob et al., 2000; Jacob et al., 1998).

The biological significance of LPO lies in its role as a natural defense system against microbial invasion. Bovine milk is the only milk containing antimicrobial agents. In addition to its antiviral effect, LPO has been reported to provide protection against various damages and peroxidative effects in animal cells (Reiter and HaÈrnulv, 1984; Reiter and Perraudin, 1991; Wolfson and Sumner, 1993; de Wit and van Hooydonk, 1996). LPO is the most crucial enzyme in milk (Pakkanen and Aalto, 1997; Scammel, 2001). The enzyme LPO catalyzes the antibacterial oxidation of thiocyanate ions as seen in the following reaction (Kumar and Bhatla, 1995).



The LPO enzyme also catalyzes the oxidation of iodide by hydrogen peroxide (Bayse et al., 1972).



The X-ray crystallographic structure of the LPO enzyme is depicted in Figure (Each protein chain is shown in a different color. Additionally, the heme group is represented in white, nitrogens in blue, and the iron group in orange (Singh et al., 2007).

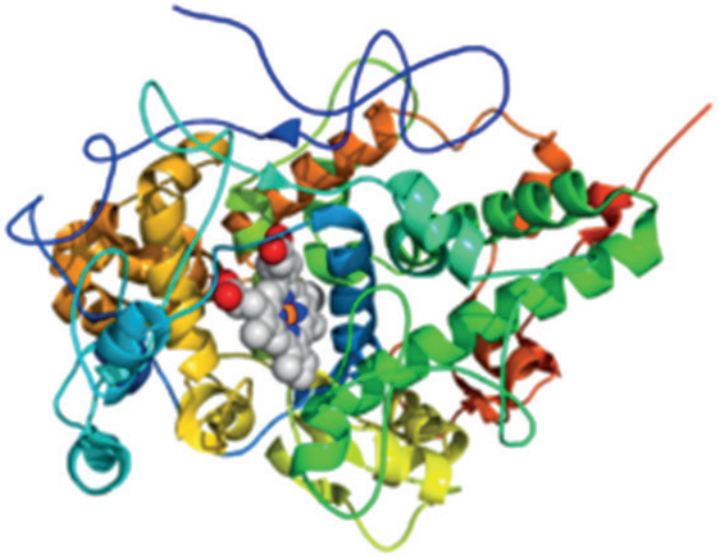


Figure 1.2. *X-ray Crystallographic Structure of the LPO Enzyme*

The hem group is covalently bound to the protein, and this binding occurs between the hydroxyl group of the hem group and the carboxyl group of the protein (Doumonted and Rousset 1983). Mammalian milks, due to containing antimicrobial factors such as lysozyme, lactoferrin, immunoglobulins, and LPO, have high biological value (Elagamy et al. 1992).

LPO is a member of mammalian peroxidases activated in various anatomical parts, utilizing hydrogen peroxide to oxidize thiocyanate to hypothiocyanate. The catalytic center's hem group is protoporphyrin IX, covalently bound to the polypeptide chain through a disulfide bridge (Thanabal and La Mar 1989). Like other peroxidases, LPO catalyzes the oxidation of molecules such as phenols, aromatic amines, pyrogallol, ascorbate, and guaiacol in the presence of H2O2 (Doumonted and Rousset 1983, Kumar and Bhatla 1995).

In a study conducted with the presence of H2O2, the mechanism of guaiacol oxidation was elucidated, and it was found that guaiacol is oxidized to 3,3'-dimethoxy-4,4'-biphenyloquinone (Daniel et al. 1997).

Among its most common substrates are simple phenols such as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), guaiacol, and catechol (Shindler and Bardsley 1975, Daniel et al. 1997, Metodiewa et al. 1989). It is also known to be inhibited by hydrazines (Kumar and Bhatla 1995). Some thiocarbamide compounds have been identified to inhibit bovine LPO enzyme (Doerge 1986).

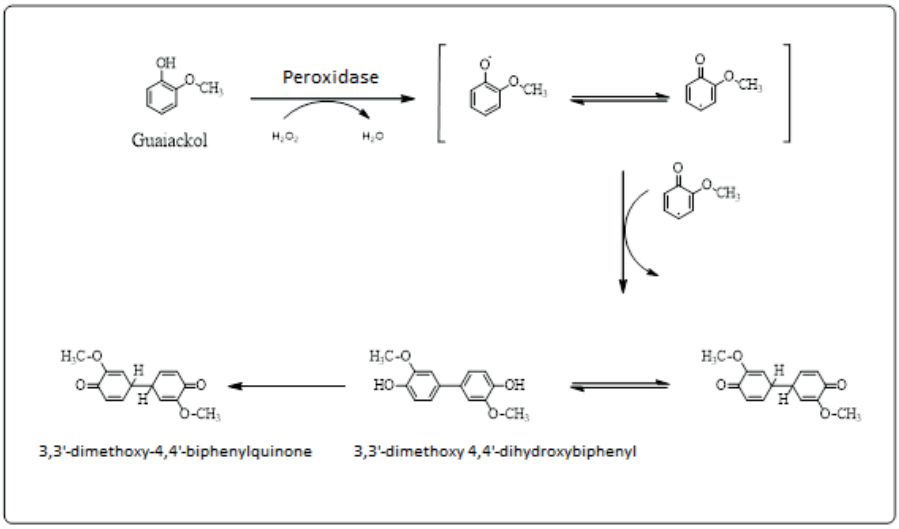


Figure 1.3. *Mechanism of the Peroxidase Enzyme Converting Guaiacol Substrate into the Colored Oxidation Products of Guaiacol*

The reaction mechanism, where hydrogen peroxide is reduced and guaiacol substrate is oxidized, occurs in three steps;

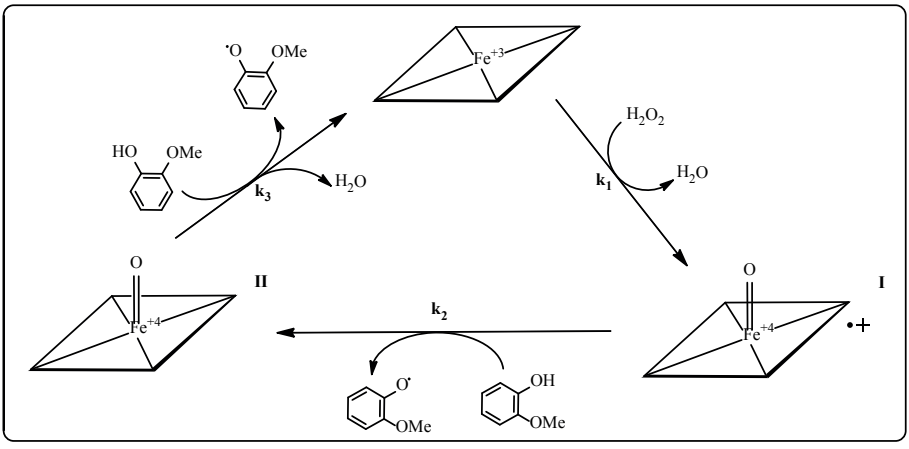


Figure 1.4. *Reaction Catalysis Cycle of Peroxidase Enzyme in the Presence of Guaiacol Substrate*

In the initial step of this mechanism, LPO enzyme reacts with an equivalent of peroxide to form compound I, which contains Fe(IV) in the porphyrin cation radical. This redox reaction involves the reduction of hydrogen peroxide to water, leading to oxidation of the enzyme. In the second step, compound I, in its cation radical form, is reduced to Fe(IV) by accepting a proton from the substrate (guaiacol) in the environment, while the substrate loses a proton and returns to its radical form. The resulting compound II

is a porphyrin with an oxoferryl center. In the final step, compound II is reduced back to its initial state by accepting another proton from the substrate (guaiacol). Additionally, radical substrates formed in the environment polymerize through interactions with each other (Veitch 2004).

Numerous research groups aim to purify LPO enzyme and study its inhibitors (Özdemir and Uguz 2005; Özdemir et al. 2001; Shin et al. 2001). Different chemicals acting as inhibitors have been studied on bovine LPO, and many have been shown to inhibit the enzyme (Özdemir et al. 2002, 2003; Özdemir and Uguz 2005; Sisecioglu et al. 2011). The first purification method used in enzyme purification is based on precipitation with salt concentration. Ammonium sulfate is a commonly used neutral salt in this method (Özdemir et al. 2001, 2003). Chromatographic methods, including ion exchange, bioaffinity, and hydrophobic affinity chromatography, have been employed. The initial steps for these methods involve cell disruption and centrifugation. Molecular weight is calculated using the gel filtration method (Voet and Voet 1959).

The purification and characterization of LPO enzyme from bovine milk have been investigated by many research groups. Studies have explored various aspects, such as the structural characteristics of the prosthetic heme group in bovine milk LPO (Rae and Goff 1998), the effects of surfactants on bovine LPO activity stabilization (Marcozzi et al. 1998), the impact of lactation days on LPO system components in goat milk (Zapico et al. 1991), norepinephrine peroxidative oxidation by LPO (Metodiwa et al. 1989), studies on the mechanism of tyrosine iodination by LPO (Huber et al. 1989), potentialization of the LPO system for preserving raw milk at tropical temperatures (Paricio et al. 1986), the effect of LPO on lipolysis and lipoprotein lipase activities in milk (Ahrne et al. 1985), structural investigation of milk LPO using circular dichroism and various absorption spectroscopy methods (Sievers 1980), and the peroxide-catalyzed singlet oxygen production by indole-3-acetic acid (Kanofsky 1988), among others.

The purification of LPO enzyme from bovine milk and its characterization have been reported in the literature using various methods. For instance, CM-Sephadex ion-exchange chromatography (Özdemir et al. 2003; Uguz and Özdemir 2005), Sephadex G-100 gel filtration chromatography (Özdemir and Uguz 2005; Shin et al. 2001), hydrophobic affinity chromatography on Phenyl-Sepharose CL-4B (Langbakk and Flatmark 1984), and cation exchange chromatography on Toyopearl-SP (Mecitoglu and Yemenicioglu 2007) are among the methods used for the purification of LPO enzyme from bovine milk. All studies indicate that the purification of LPO is time-consuming and can be achieved with complex methods (Mecitoglu and Yemenicioglu 2007). Purification has been reported with yields of 31-fold at 64%, 10-fold at 45%,

and 13.2-fold at 29% in various studies (Nandini and Rastogi 2010; Shin et al. 2001).

In a study conducted in 1983 by Doumonted and Rousset, a purification method for LPO was established. Initially, they removed casein from raw bovine milk by adding 10 mg of rennet per liter and shaking until coagulation occurred at room temperature. All steps other than this were carried out at +4°C. They centrifuged the coagulated material at 2600xg for 15 minutes and removed the obtained pellet. For each liter of the supernatant obtained, they added 40 ml of water-washed Amberlite CG-50 (in NH4+ form) and stirred for 3 hours. They then washed the obtained Amberlite CG-50 pellet first with water and then with 50 mM sodium acetate buffer. After washing, they loaded the pellet onto a column and eluted with 500 mM acetate buffer.

They precipitated the obtained eluates with ammonium sulfate and centrifuged the solution at 3000xg for 30 minutes. The obtained pellet was dissolved in 100 mM sodium acetate buffer. Thus, both heme-containing and heme-free crude LPO were obtained. In the next step, this obtained product was loaded onto a Sephadex G-25 column balanced with 100 mM acetate buffer, and ammonium sulfate was removed from the environment. The second purification step involved loading the product onto an Amberlite CG-50 column and eluting with a gradient of 100 mM to 1M sodium acetate. This separated heme-containing LPO from heme-free LPO.

The final product obtained was stored in 3.2 mM acetate buffer (Doumonted and Rousset 1983).

In another study, degreased bovine milk was first stirred with Amberlite CG-50 (4.4 g/150 mL) for an hour. Afterward, the material was washed with water and then with a 20 mM sodium acetate buffer to obtain crude LPO using a Buchner funnel. The obtained product was precipitated with 90% ammonium sulfate saturation, and the pellet was centrifuged at 3000xg for 15 minutes. The resulting product was loaded onto a CM-Sephadex column and eluted with a gradient of 10 mM phosphate buffer containing 100 mM-200 mM NaCl. They subjected the eluates to ammonium sulfate precipitation each time with 90% saturation. The product obtained after ammonium sulfate precipitation was finally loaded onto a Sephadex G-100 column, and elution was collected with a 100 mM phosphate buffer. The obtained LPO enzyme was stored in a 1M phosphate buffer at pH=6.8 (Siseciogl u et al. 2010).

Sulfanilamide compounds (R-SO2-NH2) contain heterocyclic aromatic imines with acidic nitrogen groups, including histidine and imidazole (Drew 2000). Sulfanilamide finds applications in the treatment of various bacterial diseases in humans and other species, as well as in promoting the growth of food-producing animals. Sulfanilamides represent a significant class of drugs

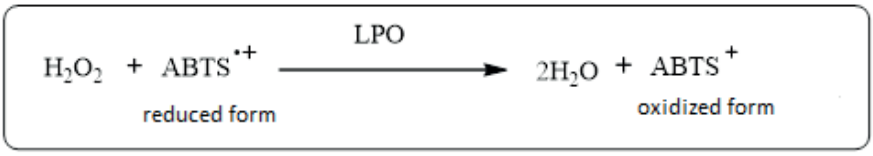
with pharmacological agents exhibiting antibacterial, antiglaucomatous, diuretic, hypoglycemic, and antithyroid effects (Supuran 2002). Structural studies indicate that new sulfanilamide derivatives possess protease inhibitor properties (Supuran 2003).

Attaching sulfanilamide to cyanogen bromide-activated Sepharose-4B is an easy method. The purification of LPO enzyme from sulfanilamide compounds using the affinity technique has not been reported using cyanogen bromide-activated Sepharose-4B to date. In the latest purification technique applied to LPO, single-step purification was achieved using the affinity technique, with sulfanilamide utilized as the ligand (Atasever et al. 2013).

In the conducted study, the kinetic properties of sulfanilamide compounds on LPO were reported for the first time. Sulfanilamide was identified as a potent and competitive reversible inhibitor of LPO. The Ki value was determined to be 3.55x10-5 M, and the IC50 value was reported as 0.848 mM, indicating that the enzyme was purified 428.57-fold (Atasever et al. 2013).

Determination of Lactoperoxidase Activity:

The measurement of LPO enzyme activity was carried out using a procedure based on modifications of the method developed by Shindler and Bardsley (1975). This method relies on the oxidation of the chromogenic substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) by H_20_2, and the resulting increase in absorbance is monitored at 412 nm (Shindler and Bardsley, 1975).



For the activity determination, the following procedure was followed: 2.8 ml of 1 mM ABTS and 0.1 ml of 3.2 mM H_20_2 were pipetted into a 3 ml spectrophotometer cuvette. Then, 0.1 ml of the enzyme solution was added, and the chronometer was started. After inverting the cuvette, it was immediately placed into the spectrophotometer, and the increase in absorbance at 412 nm against the blank was recorded every 15 seconds for a duration of 3 minutes. Each activity measurement was repeated three times. As a blank, 0.1 M phosphate buffer at pH=6 was used instead of the enzyme solution, and other solutions were used in the same proportions. The following formula was used for activity determination:

$A = \varepsilon.b.c \quad c = A/\varepsilon.b \quad V = c.D_f$

A = Absorbance (read after 1 minute)

b = Path length of light (1 cm)

c = Concentration (μmol/ml)

ε = Extinction coefficient (32400 M^{-1} x cm^{-1})

D_f = Dilution factor

V = Reaction rate (μmol x min. /ml)

In the enzyme purification processes, ABTS will be used as a substrate; therefore, the results of the enzyme activity measurements are defined as follows: 1 enzyme unit is defined as "the amount of enzyme catalyzing the oxidation of 1 μmol of ABTS in 1 minute at 20°C."

CONCLUSION:

Enzyme kinetic studies represent a crucial area in biochemistry and molecular biology, with significant implications for understanding biological processes, drug development, and cellular regulation.

LPO is an enzyme that oxidizes organic substrates in the presence of peroxides. This enzyme is particularly found in milk and secretory fluids. Investigating the kinetic properties of the LPO enzyme can contribute to a better understanding of its role in biological processes. LPO, especially present in milk, may contribute to the immune system with its antimicrobial activity. The enzyme's application in the food industry is essential, potentially enhancing the quality and durability of dairy products. The ability of LPO to oxidize substrates can contribute to its antimicrobial activity. These kinetic studies can aid in understanding the interaction mechanisms of LPO with bacteria and other microorganisms.

Understanding the mechanisms that make LPO a potential targeted drug can be facilitated by these studies. Determining the profile of reactants and products used in the reactions catalyzed by LPO can provide insight into the enzyme's specificity and efficiency. This information contributes to understanding the potential use of LPO as a targeted drug. The translation of LPO's catalyzed reactions into therapeutic applications may involve understanding its specificity and effectiveness.

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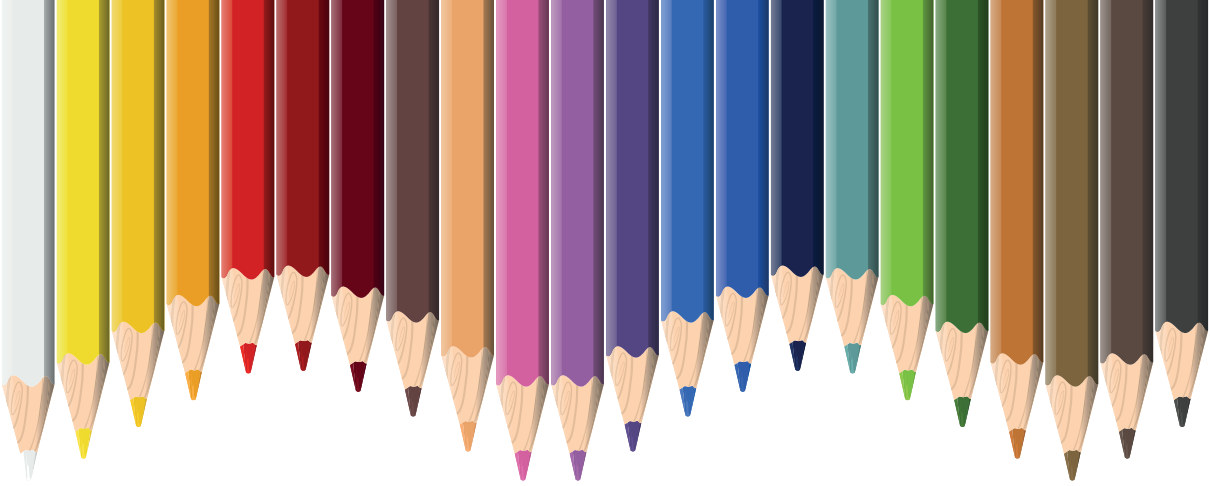

Chapter 2

THE EFFECTS OF DECOMPOSERS IN MARINE ECOSYSTEMS

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GENERAL DEFİNİTİON OF MARİNE ECOSYSTEMS

One of the most striking features that distinguishes Earth from the rest of the universe is the presence of life. This gives our planet a unique quality. This characteristic also directs the functioning of Earth's ecosystems. Scientifically, the diversity of life is noteworthy, and there are approximately 1.9 million different organism species identified on Earth. Moreover, at least 226,000 of these organisms exist in the oceans. It is believed that there are many more species than those that have been identified to date. Based on various methods, it is estimated that there are between 5 to 11 million eukaryotic species on our planet, and of these, 0.7 to 2.2 million species are thought to live in the oceans (Appeltans et al. 2012; Costello et al. 2012; Pimm et al. 2014). While the life of all living beings on Earth depends on water, soil, and air, the importance of water is greater compared to others due to about 75% of the Earth being covered with water and a large portion of living beings' bodies consisting of water. The Earth's water resources are made up of oceans, seas, lakes, rivers, glaciers, and groundwater. Of these resources, 97% are saline, and 3% are freshwater (Yıldız et al. 2005). An ecosystem is defined as the entirety formed by biotic (living) organisms (plants, animals, microorganisms) that reside in a certain area and interact with each other and their environment, and abiotic (non-living) factors (soil, water, air) (Kocataş, 2014; Weathers et al. 2021). While the life of all living beings on Earth depends on water, soil, and air, the importance of water is greater compared to others due to about 75% of the Earth being covered with water and a large portion of living beings' bodies consisting of water. The Earth's water resources are made up of oceans, seas, lakes, rivers, glaciers, and groundwater. Of these resources, 97% are saline, and 3% are freshwater (Yıldız et al. 2005). An ecosystem is defined as the entirety formed by biotic (living) organisms (plants, animals, microorganisms) that reside in a certain area and interact with each other and their environment, and abiotic (non-living) factors (soil, water, air) (Kocataş, 2014; Weathers et al. 2021).

Figure 1. *Map of the World's Oceans and Seas (Abubakar, 2007).*

DECOMPOSITION IN MARINE ECOSYSTEMS

Decomposers, widely distributed in the world's blue waters, play significant roles in the ecological food chain. They are known as the 'cleaners' of the ecosystem due to their ability to break down complex organic materials into simpler forms. Unlike terrestrial organisms, the marine flora and fauna have adapted to marked environmental changes as they are exposed to varying degrees of environmental factors such as pressure, light, salinity, and oxygen levels (dissolved oxygen). For the continuation and existence of life, every level in the food chain must be in balance. Therefore, just as it is important for phytoplankton to provide energy to higher-level consumers in the marine food web, the decomposition of dead organic matter and the breakdown of nutrients into forms usable by phytoplankton are also critically important. Decomposers play a crucial role in cleaning the ocean biome as well.The seas are a habitat for various groups of microorganisms such as bacteria, filamentous fungi, yeasts, microalgae, and protozoa, and these organisms live in diverse types of niches. They are distributed either as neuston (also known as pleuston) at the sea surface, as plankton in the photic zone of the pelagic region, or in epibiotic habitats (as attached communities) (Mitra and Zaman 2016).

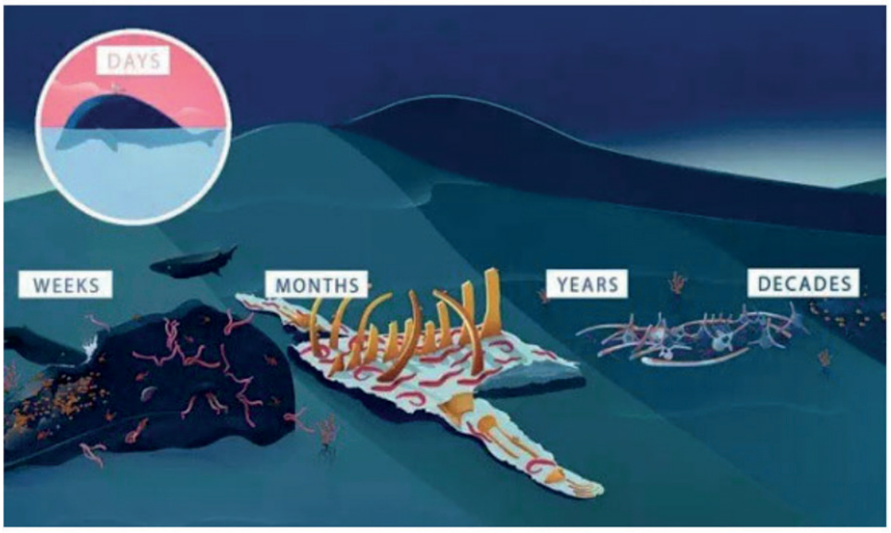

Figure 2. *Decomposition of a dead whale body in an ecosystem (Winters 2019).*

Marine microorganisms, being the most common forms in the marine ecosystem, live everywhere from the deepest trenches to the highest tidal areas. Despite being very small and simple in structure, microorganisms, which have played significant roles in the evolution of life on our planet, are essential for the existence of life on Earth and for supporting other life forms. These organisms are often the most important primary producers in many marine ecosystems, directly or indirectly feeding most marine animals. Some microorganisms provide basic nutrients to primary producers, either initially or by recycling them. Others float in the water, consuming food like animals, and play critical roles in marine food webs (Castro and Huber 2008).

Marine Bacteria

Bacteria, which are prokaryotic and simple in structure, share similarities with the Archaea domain. Prokaryotic cells do not possess organelles enclosed by membranes (such as nuclei, mitochondria, or chloroplasts). Bacterial cells contain a single circular DNA chromosome that carries a relatively small number of genes, and they are surrounded by a lifeless cell wall that provides support and protection. They reproduce asexually through division. Bacteria, with their diverse and unique metabolic abilities, along with their abundant presence and wide distribution in the seas, are indispensable components of marine ecosystems. Marine bacteria are primary producers, decomposers, active participants in biogeochemical cycles, a source of nutrition for other marine organisms, modifiers of marine sediments, and organisms that can establish symbiotic relationships as well as cause diseases. Bacteria achieve locomotion through a protein motor powered by protons and consist of a

twisted filament that rotates like a corkscrew. Bacteria come in a wide variety of shapes and sizes and are categorized as coccus (spherical), bacillus (rod-shaped), and the least common in marine environments, spirillum (corkscrew-shaped). Apart from these, there are also marine actinobacteria, which have a more fungus-like appearance and live in sediments. Marine bacteria are generally a few micrometers in length or width. Bacteria in marine ecosystems perform multiple roles and acquire nutrients in various ways. Some marine bacteria that produce organic molecules from inorganic ones are primary producers (autotrophs). In photosynthetic organisms, the energy source for primary production is the sun, whereas for those performing chemosynthesis, it is simple and abundant chemicals. Other bacteria, which need organic molecules for nourishment and use these molecules to produce additional bacterial biomass, are heterotrophs. Heterotrophic marine bacteria that take in organic materials from the external environment through absorption across their cell walls and membranes engage in a form of nutrition known as osmotrophy. Bacteria feeding in this manner secrete digestive enzymes (extracellular enzymes) to break down large organic molecules into smaller fragments for absorption. Heterotrophic bacteria are decomposers because of their ability to convert dead organic matter into living bacterial biomass and release usable inorganic molecules for primary producers in biogeochemical cycles. One of the most important metabolic capabilities of bacteria is their ability to break the strong bond of molecular N_2 and convert it into ammonium ion, a form usable by other living organisms. With these characteristics, they support life in many marine habitats, which otherwise would lack sufficient nitrogen for life (Kirchman 2008; Nagata 2008; Emerich and Krishnan 2009; Karleskint et al. 2012).

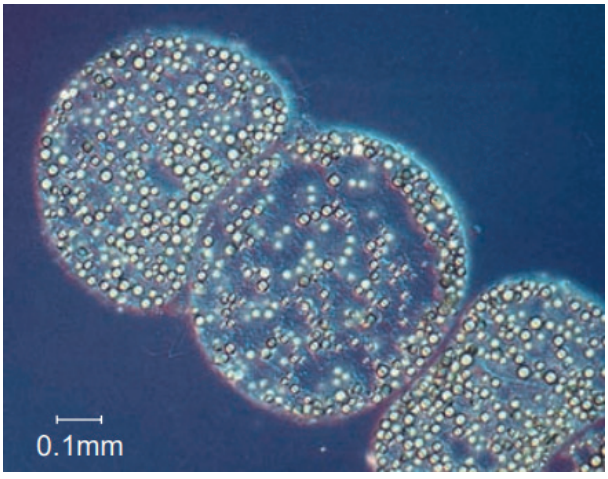

Figure 3. *The largest known bacteria Thiomargarita namibiensis, lives in low-oxygen waters (Castro and Huber 2008).*

Marine Heterotrophic Bacteria

Organisms that decompose organic materials in their environment for the synthesis of compounds and for energy utilization are called heterotrophic bacteria. The respiration and fermentation carried out by these bacteria result in the release of inorganic chemicals back into the environment, making them usable for primary producers. Heterotrophic bacteria decompose cellulose, lignin, chitin, keratin, and other resistant natural molecules through the exoenzymes they secrete. Recent studies have shown that enzymes released by bacteria can also degrade plastics and synthetic polymers in the oceans. Without heterotrophic bacteria, complex substances would accumulate in the seas, becoming unusable as nutrients for other organisms for thousands of years. Organic materials in the sea are mostly found as particles suspended in the water or accumulated on the ocean floor. These particles become quickly colonized by bacteria as soon as they form. The release of exoenzymes outside the cell brings the risk of dilution in the surrounding environment. To combat this risk, bacteria secrete mucus that adheres them to the particles. The mucus keeps the bacteria near their food source where they secrete exoenzymes, ensuring that the digestible materials do not drift away from them. Different species of bacteria on the same food particle provide a variety of enzymes, creating a broad diversity of nutrients and thus bringing forth more food for absorption. Bacteria's interaction with particles in the marine environment supports three main processes: consolidation, lithification, and sedimentation. The first process, consolidation, involves the secretion of mucus and alteration of the electric charge of particles, resulting in sticky surfaces that cause adjacent particles to adhere to each other. The second process, lithification, occurs as bacteria's metabolic activity changes the acidity or alkalinity (pH) around the particle, leading to the precipitation of minerals and the formation of a cement-like substance between the particles. The final process, sedimentation, is observed as particle aggregation increases due to consolidation and lithification. As the aggregate size increases, the water cannot suspend the particles anymore, and they begin to settle. Particles can accumulate on the ocean floor, at the convergence zones of water layers of different densities, or within large networks formed by mucus secreted by plankton (marine snow). As these particles start accumulating, they become available for suspension and detritus feeders. These consumers feed on the large microbial communities that grow on the particles (Caron et al. 1982; Ramaiah 2004; Robinson 2008; Karleskint et al. 2012).

Marine Cyanobacteria

Genetically and ecologically, the best-understood group among marine bacteria is likely the marine cyanobacteria. Most members of this group have been cultured, and their genomes have been completely sequenced.

Additionally, all identified members of this group carry chlorophyll a and perform oxygenic photosynthesis, which is something that no other bacteria or archaea can do. The cyanobacteria genus Prochlorococcus, first discovered using flow cytometry, is now known to be among the most abundant (Chisholm et al. 1988). The small cells of Prochlorococcus, approximately 0.6 mm in diameter, remained undetected by epifluorescent microscopy for many years until flow cytometry was used. Their pigments (divinyl chlorophyll a and b) did not exhibit bright enough fluorescence for detection and photosynthetic identification under epifluorescent microscopy. Synechococcus, a close relative of Prochlorococcus, has cells approximately 0.9 mm in size. It was widely detected in seawater in a study conducted in 1979 (Johnson and Sieburth 1979; Waterbury et al. 1979). However, Synechococcus is much easier to detect because its phycoerythrin accessory pigment emits a bright yellow-orange autofluorescence under blue or green excitation. Despite being similar in size and closely related from a phylogenetic perspective, most Prochlorococcus and Synechococcus are thought to have different ecological strategies. While Prochlorococcus is more commonly found in oligotrophic waters, Synechococcus is more dominant in waters with higher nutrient levels. Synechococcus, often described as more generalist, is reported to be more capable of taking advantage of fluctuating environments (Rocap et al. 2002; Palenik et al. 2003; Dufresne et al. 2005). Prochlorococcus, which has multiple ecotypes, exhibits its most distinct variations in adaptations to high light and low light, divided by depth and environmental gradients (Rocap et al. 2002; Johnson et al. 2006). The high light-adapted ecotype of Prochlorococcus is known as the smallest genome of any oxygenic photosynthetic organism, with a strain having approximately 1.7 million base pairs in its genome. Similar to some Synechococcus strains, the low light-adapted strains can have genomes of approximately 2.4 million base pairs (Rocap et al. 2002; Palenik et al. 2003). In addition to Synechococcus and Prochlorococcus, other cyanobacteria also have a special importance in marine ecosystems due to their roles in the fixation of atmospheric nitrogen. For example, the colony-forming Trichodesmium, often visible to the naked eye as tufts or clumps, predominantly inhabits tropical and subtropical waters. While generally rare, it can have intense episodic local blooms and is thought to contribute significantly to the global nitrogen pool (Capone et al. 1997, 2005).

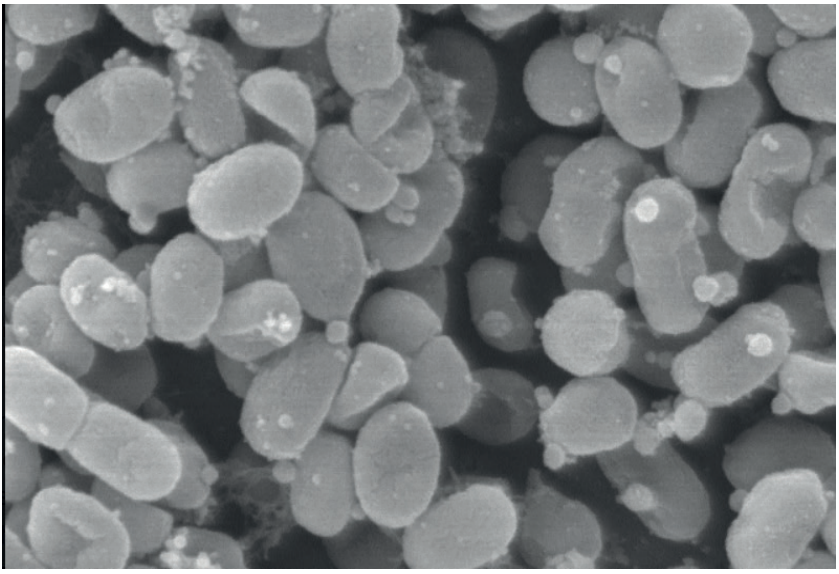

Figure 4. *Scanning electron micrograph showing marine cyanobacteria Prochlorococcus with small vesicles visible near the cell surface (Biller et al. 2014).*

Cyanobacteria affect the production of marine products and the nutrient cycle in two ways. Firstly, they play an important and sometimes dominant role in the fixation of carbon dioxide (CO_2) through photosynthesis, contributing significantly to the carbon cycle and primary production. Examples include picoplanktonic genera such as Synechococcus, Prochlorococcus, and Synechocystis. These forms are responsible for a large portion of phytoplankton biomass and primary production in waters ranging from oligotrophic open oceans to more eutrophic coastal and estuarine ecosystems. Secondly, some cyanobacterial genera can convert dinitrogen into ammonia (NH_3), a biologically usable form, through nitrogen fixation (Fogg, 1944, 1974; Stewart, 1973). This is a relevant and important process for ocean areas showing nitrogen deficiency (Dugdale and Goerinc 1967). Ammonia alleviates N-limited growth and serves as a new N source to support the primary and secondary production of microbes, higher plants, and animals. The nitrogen fixation capability has allowed certain marine planktonic cyanobacteria genera to proliferate as intense surface blooms in oceanic, coastal, and estuarine waters that are chronically deficient in N. Diazotrophic cyanobacteria play significant roles in mutual and symbiotic relationships with microalgae, macroalgae, as well as with rooted plants and animals (Carpenter, 1973; Stewards, 1973; Capone 1983; Phlips and Zeman 1990; Gallon et al. 1992; Villareal, 1992).

Marine Chemosynthetic Bacteria

Chemosynthetic bacteria in the lightless areas of ocean waters and sediments use inorganic chemicals instead of light for autotrophic energy. These bacteria utilize the energy derived from chemical reactions involving substances such as ammonium ion (NH_4^+), sulfides (S^{-2}) and elemental sulfur (S), nitrites (NO_2^-), hydrogen (H_2), and ferrous ion (Fe^{+2}). The energy obtained is mostly used to produce organic nutrient molecules using carbon dioxide (CO_2) as a carbon source. Chemosynthesis is much less efficient than photosynthesis, resulting in slower cell growth and division rates when large amounts of compounds are not present in the environment. Therefore, chemosynthetic bacteria are found in places where there is an abundance of the inorganic materials they require. Additionally, these bacteria are mostly anaerobic. There are some chemosynthetic bacteria that can live around deep-sea hydrothermal vents. These bacteria sustain their lives as microbial mats in or near the vents, in high concentrations in the waters over the hot vents, where they are consumed as food by suspension feeders and benthic grazers. They use the energy generated from transforming leaking sulfur ions into sulfur and sulfate, also consuming it as nutrients. These chemosynthetic bacteria form the base of a productive food chain in the hydrothermal vent community, comprising a diverse array of organisms including worms, mussels, and crabs. Others live symbiotically with benthic invertebrates (Karleskint et al. 2012).

Marine Fungi

Fungi, while being multicellular organisms, also include some unicellular forms such as molds and yeasts. They lack chloroplasts and chlorophylls and are non-photosynthetic heterotrophs. There are known to be about 1500 marine fungal species, most of which are microscopic in nature. Ascomycetes are the most diverse and abundant phylum of marine fungi, comprising 81% of the less than 500 known species of marine filamentous fungi. Additionally, many of the approximately 200 marine yeasts are also ascomycetes. Many fungal species decompose dead organic matter. For example, they are major decomposers of mangrove leaves, thereby contributing to the nutrient recycling in mangrove forests. Some fungal species live as parasites on marine grasses or as borers in the shells of mollusks. Others are parasites that cause diseases in economically important seaweeds, sponges, shellfish, and fish. Additionally, some marine fungal species are being researched as a source of antibiotics for medical use. Moreover, certain types of marine fungi establish symbiotic relationships particularly with green algae or cyanobacteria, forming unique entities known as lichens. Lichens are supported nutritionally by fungi, while algae or cyanobacteria meet their needs through photosynthesis. Lichens can exist as thick, dark brown or black patches in areas affected by waves on open rocky shores. Although resistant to prolonged exposure to air, there are fewer species of marine lichens compared to terrestrial ones. Some marine lichens are known to have nitrogen-fixing cyanobacteria, but there are still unexplored

aspects of their role in the ecology of rocky shores (Castro and Huber 2008; Karleskint et al. 2012; Kohlmeyer and Kohlmeyer 2013; Raghukumar 2017).

RESULT

Bacteria, microorganisms, and fungi that contribute to decomposition in marine ecosystems play a crucial role for the health and future of the seas. These organisms assist in maintaining the fundamental functions of marine ecosystems, and are critically important for the balance of marine environments. The decomposers in the marine ecosystem play a critical role in breaking down dead plant and animal matter, recycling these organic materials back into the ecosystem as nutrients and energy. They contribute to the cycling of essential nutrients such as carbon, nitrogen, and phosphorus, supporting the productivity and sustainability of the ecosystem. A balanced decomposer community positively impacts biodiversity and interactions among different species, serving as an indicator of overall ecosystem health. Furthermore, these activities are key components of the global carbon cycle, and their contributions to the carbon cycle can help mitigate the effects of climate change. The roles of decomposer organisms in marine ecosystems are of vital importance for the future of oceans and seas. Understanding and preserving the activities of these organisms are critically important for maintaining the health of marine ecosystems and sustaining the benefits that people derive from these ecosystems.

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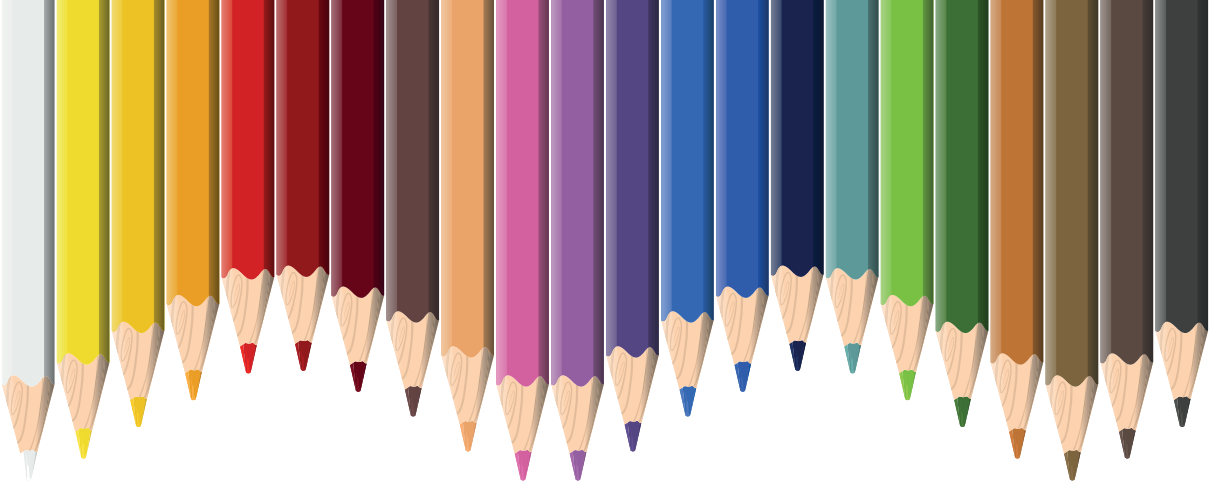

Chapter 3

GREEN SYNTHESIS AND CHARACTERIZATION OF METAL NANOPARTICLES FROM SOME SIDERITIS TYPES

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INTRODUCTION

The chemicals used in nanoparticle production have been observed to increase particle toxicity and reactivity, leading to undesirable adverse effects on human health. The limitations encountered in traditional methods and the growing awareness of human health and environmental issues in recent years have prompted the development of more sustainable methods for nanoparticle production. This has brought "green nanotechnology," which meets sustainability goals, to a highly intriguing position in today's context (Hussaın et al., 2016).

In this method, there is no need for the use of toxic chemicals and solvents (Alkhalaf, 2020). With the green synthesis method, nanoparticles with system carrier properties such as antineoplastic, antibacterial, antifungal, and antiviral characteristics can be easily obtained. Additionally, this method allows for the elimination of the toxic effects caused by chemical methods and the energy and time loss in physical methods.

Green nanotechnology is an approach that aims to reduce metal ions through the intracellular and extracellular metabolites of biological organisms and stabilize them by coating them with biological polysaccharides (Singh et al., 2016). The majority of biological organisms are capable of storing and detoxifying metal salts within their cells, thanks to the redox enzymes they possess (Singh et al., 2016). With these properties, they can reduce metal ions taken into the cell and convert them into metal nanoparticles. Additionally, it is possible to reduce metal ions to metal nanoparticles in the extracellular environment using crude extracts containing reductase enzymes or purified reductase enzymes.As an alternative to traditional methods, in the biological synthesis approach, nanoparticle synthesis is possible without the need for reducing and stabilizing chemicals.

1. Advantages of Green Synthesis

Studies on physical and chemical methods have revealed that the physicochemical properties of metal-based nanoparticles (NPs), such as size, morphology, stability, and reactivity, are strongly influenced by experimental conditions, the interaction kinetics between metal ions and reducing agents, and the adsorption between stabilizing agents and metals (Sharma et al., 2009). Despite the lower efficiency compared to other methods under current conditions, green synthesis is widely preferred due to its energy efficiency and environmentally friendly conditions, avoiding the use of toxic chemicals and solvents (Mohanpuria et al., 2008).

The biological production of highly stable and well-characterized NPs can be achieved by optimizing parameters such as organism types, cell growth,

enzyme activity, optical growth, reaction conditions, and suitable biocatalysts. Green synthesis, despite its lower efficiency under current conditions, is favored because it makes the process safer by eliminating the need for energy and using environmentally friendly conditions, avoiding toxic chemicals and solvents (Mohanpuria et al., 2008).

The biological production of highly stable and well-characterized nanoparticles (NPs) can be achieved by optimizing parameters such as organism types, cell growth, enzyme activity, optical growth, reaction conditions, and suitable biocatalysts (Shankar et al., 2004). Additionally, NPs synthesized through green methods exhibit higher antimicrobial activity compared to those synthesized by other methods. It is believed that the synergistic effect of several proteins that play a role in encapsulating and subsequently stabilizing biosynthesized NPs contributes to this antimicrobial activity (Roy et al., 2019).

The fundamental properties of NPs, such as electronic, optical, magnetic, and catalytic characteristics, are controlled by the size and shape of the NPs. Due to the ease of controllability in biological systems, the green synthesis of NPs is considered a more favorable method than chemical synthesis (Ghorbani et al., 2011).

1.2. Factors Influencing Nanoparticle Synthesis with Plant Extracts

The formation of nanoparticles through the reduction process of metal ions is influenced by various factors, including the composition of a plant extract containing different bioactive molecules, as well as factors such as pH, temperature, reaction time, concentration, and the electrochemical potential of a metal ion. (Makarov et al., 2014).

1.2.1. pH

It plays a significant role in the green synthesis of nanoparticles. A change in pH induces a charge alteration in the natural phytochemicals present in the extract, affecting their ability to bind to and reduce metal cations and anions during nanoparticle synthesis. This, in turn, influences the shape, size, and yield of the nanoparticles. (Makarov et al., 2014)

1.2.2. Temperature and Reaction Time

It is another crucial factor influencing nanoparticle formation from plant extracts. Generally, an increase in temperature enhances the reaction rate and efficiency of nanoparticle synthesis. The size, shape, and scope of nanoparticle synthesis using plant-based biomaterials are also significantly affected by the duration of the reaction time during which the synthesis environment is incubated. (Vijayaraghavan et al., 2017).

1.2.3. Amount of Plant Extract

It affects the efficiency of nanoparticle synthesis. Previous studies have shown that an increase in the amount of extract not only enhances the synthesis of nanoparticles but can also alter their morphology. Therefore, determining the optimum extract amount for synthesis is often crucial. (Vijayaraghavan et al., 2017).

1.2.4. Electrochemical Potential of the Metal Ion

It is considered that the ability of plants to reduce metal ions is limited, and the efficiency of metal nanoparticle synthesis is also dependent on the electrochemical potential of the metal ion. Ions that are more easily reducible are more suitable for nanoparticle synthesis, and the effective reduction capacity of the plant is larger for such ions. (Haverkamp et al., 2009).

2. Biological Agents Used in Biological Synthesis

For the green synthesis of nanoparticles, biological organisms such as microorganisms, fungi, lichens, algae, and plants can be used. This involves using crude extracts containing intracellular metabolites from these biological organisms, or purified biomolecules such as enzymes, pigments, polysaccharides, as reducing and stabilizing agents. (Shah et al., 2015).

2.1. Microorganisms

The biological synthesis of nanoparticles is mediated by biomolecules such as enzymes and proteins produced by microorganisms (Shah et al., 2015). Microorganisms can alter the state of metal ions, allowing for their accumulation and creating the potential for particle synthesis (Babitha et al., 2013). Previous studies have demonstrated the formation of nanoparticles both inside and outside the microbial cells. Bacterial cultures are prepared in complex environments with factors such as light, nutrients, temperature, and pH. (Shah et al., 2015).

2.2. Fungi and Lichens

Fungi, due to the presence of reducing agents within the cell wall and their high content of enzymes and proteins, are highly efficient in facilitating nanoparticle synthesis (Shah et al., 2015). Additionally, saprophytic fungi exhibit a high rate of production (Deepak 2014). Fungi are more economical compared to bacteria. Their high accumulation property and metal ion tolerance provide several advantages in nanoparticle utilization (Baskar et al., 2013). Throughout the entire synthesis process, proteins and enzymes are utilized as both coating and reducing agents.

2.3. Algae

Algae, being inexpensive, effective, and environmentally friendly, are impactful in the agriculture, pharmaceutical, medical, and cosmetic sectors in nanoparticle synthesis (Hulkoti et al., 2014). Marine algae are highly rich in oil, protein, phenolic compounds, and minerals. The reduction and coating of metals utilize the presence of hydroxyl and carboxyl groups within algae, making them suitable for the production of nanoparticle synthesis (Mahdavi et al., 2013).

2.4. Plantae

The short duration of the nanoparticle synthesis process is achieved through the use of plant extracts (Ajitha et al., 2015). When plants are used, there is no need for toxic substances, and the production benefits from not requiring high temperature and pressure, leading to a significant advantage (Mittal et al., 2014). The plant synthesis method is much simpler and faster than other methods (Fenfen et al., 2014). Due to its economic and environmentally friendly nature, the structure and stability of crystals can be controlled (Mittal et al., 2014). In plants, compounds such as proteins, amino acids, enzymes, polysaccharides, phenolic compounds, and organic acids are used as coating and reducing agents during synthesis (Makarov et al., 2014; Raut et al., 2014). Furthermore, it is known that the use of plant extracts affects the size and morphological characteristics of nanoparticles (Kumar et al., 2009). In green synthesis processes, metals such as silver, gold, iron, titanium, and thallium are preferred for nanoparticle synthesis (Patil, 2022).

3.General Information about Sideritis Plant

The majority of studies on the Sideritis genus mainly consist of pharmacological investigations, while anatomical studies are limited, as revealed by literature reviews. Plants belonging to the Sideritis genus contain numerous secondary bioactive compounds. Studies have isolated terpenes, flavonoids, essential oils, iridoids, coumarins, lignans, and sterols from plants within the Sideritis genus. The volatile oil profiles of Sideritis plants are similar, and compounds from the flavonoid and diterpene classes found in plants of the genus typically share similar characteristics. Terpenes, flavonoids, essential oils, iridoids, coumarins, lignans, and sterols in the *Sideritis* genus are responsible for its in vivo and in vitro pharmacological effects (Santiago, 2012).

3.1. Silver Nanoparticles

Silver (Ag) is utilized as a therapeutic agent in various diseases. Silver nanoparticles (Ag NPs) can be produced through both bottom-up and top-down approaches (Ahmed et al., 2016). For the production of Ag NPs, a silver metal

ion solution and a reducing agent are required. Plants, vitamins, proteins, and amino acids are used in the production of Ag NPs. The shape of the produced particles can be modified by changing the reducing agent and solvent content. The pH of the medium can significantly alter the size of the nanoparticles. It is well-known that Ag NPs are widely used in cancer treatment. The antibacterial and antimicrobial activities of Ag NPs contribute to their effective use in the medical field. Additionally, silver exhibits high catalytic activity and antioxidant properties (Singh et al., 2010).

Several studies have been published on the biological synthesis of Ag NPs. Ag NPs with sizes less than 140 nm have been produced using different bacterial species. In a different study, Ag nanoparticles produced using olive leaves were reported to exhibit antibacterial properties. In 2022, Hance and colleagues reported the production of Ag nanoparticles using Japanese mint and suggested their potential use in cancer treatment (Gao 2022). Iqbal and colleagues produced Ag nanoparticles using the leaves of the neem tree and reported higher antidiabetic properties against gram-positive/gram-negative bacteria (Iqbal et al., 2021).

3.2. Gold Nanoparticles and Studies Conducted

Gold (Au) has a melting point of 1064 °C and a boiling point of 2808 °C. Gold exhibits exceptional conductivity (Parida et al., 2016). Gold possesses properties such as biocompatibility, low toxicity, and strong scattering and absorption capabilities. Gold is commonly used in the field of medicine. Stable gold nanoparticles can be obtained by adding a reducing agent to gold metal ions (Begüm et al., 2009). Flavonoids and phenols present in plant extracts assist in the synthesis of Au nanoparticles. Additionally, this allows the production of Au nanoparticles in various forms such as triangular, spherical, etc. (Vanholme., 2008).

Gold and silver nanoparticles find applications in electronic devices such as light-emitting diodes. Plant extracts like geranium and aloe vera have been used in the production of Au nanoparticles (Jadoun et al., 2021).

In addition, bacteria and algae are other natural agents used in the production of Au nanoparticles. Various agents such as Amla, ironhindi, and fungi have also been employed in the biological synthesis method for Au nanoparticle powder production. Powders produced through this method exhibit antibacterial, antioxidant, and anticancer properties. Gold nanoparticles display analgesic properties and play a role in determining protein-protein interactions. Various plant extracts, including Arabica coffee (Keijok et al., 2019), Kratom plant (Kumar et al., 2019), and lemon (Bhagat et al., 2020), are examples of different extracts used in the production of gold nanoparticles.

3.3. Copper Nanoparticles

Copper (Cu) nanoparticles are reduced through both physical and chemical methods, utilizing organic solvents and toxic reducing agents (Mehta et al., 2022). Due to copper's rapid oxidation, the production of stable nanoparticles is a complex process. Cu nanoparticles exhibit high antibacterial properties when in contact with bacteria due to their large surface area. There are limited studies on the synthesis of Cu nanoparticles using plant extracts and microorganisms. Cu, being a good conductor, is commonly used in electronic devices, although the prepared Cu nanoparticles often exhibit lower conductivity (Mallikarjuna et al., 2011).

In a study by Karimi and colleagues, Cu nanoparticles were produced by reducing Cu ions with aloe vera extract. They reported obtaining an average particle size of 40 nm at the 578 nm peak in UV measurements (Karimi et al., 2015). Cu nanoparticles were synthesized using Cuscuta reflexa plant for Cu+2 ions and immobilized on the surface of Graphene/MnO2 (Naghdi et al., 2018).

Mehta and colleagues synthesized Cu nanoparticles using henna leaf extract. In their study, they produced nanobiocomposite biofilms using collagen fibers and Cu nanoparticles. The utilization of this film is suitable for numerous electronic devices (Mehta et al., 2014). In a study by Sastry and colleagues, Cu nanoparticles with sizes ranging from 20 to 50 nm were synthesized using lemon and tamarind (Sastry et al., 2013).

3.4. Paladium Nanoparticles

Palladium (Pd) nanoparticles are produced through various methods such as ion exchange, thermal decomposition, and chemical reduction, primarily based on the reduction of palladium ions with a reducing agent. Pd nanoparticles have been synthesized using different extracts, including camphor tree, banana peel, tea, soybean leaf, and chlorella (Hussain et al., 2016). Additionally, Pd nanoparticles have been synthesized using tea and coffee. Furthermore, phenolics found in the extract of the helical flower can also convert palladium to zero-valent Pd (Hussain et al., 2016; Kalaiselvi et al., 2015). Pd nanoparticles exhibit high catalytic activity and a strong affinity for hydrogen. (Hussain et al., 2016; Kalaiselvi et al., 2015).

3.5. Platinium Nanoparticles

Both palladium (Pd) and platinum (Pt) are dense and expensive metals. Platinum plays a crucial role in the production and distribution of drugs. It is used in the development of chemotherapy drugs such as cisplatin and carboplatin. Additionally, platinum is employed as an antimicrobial agent and is

preferred in sensor applications (Najlaa, 2019).

3.6. Zinc oxide (ZnO) Nanoparticles

The interest of researchers has increased in recent years due to the various applications of zinc oxide (ZnO) in fields such as optics, electronics, and biomedicine. The synthesis of ZnO nanoparticles is cheap, safe, and easy. ZnO nanoparticles, with properties such as high binding energy and a wide band gap, exhibit high catalytic activity and wound healing capabilities, making them suitable for use in various areas such as optics and biomedicine. Particularly in the biomedical field, they find applications as antifungal, antibacterial, and antidiabetic agents.

Typically, zinc oxide is formed by mixing zinc oxide, zinc sulfate, zinc acetate, or zinc nitrate with plant extracts, and the color change in the solution indicates nanoparticle formation. The bacterial synthesis of ZnO NPs is time-consuming, and there are few studies on its production using algae. In the literature, the production of ZnO nanoparticles with different species such as B. Licheniformis, Lactobacillus plantarum has been reported (Agarwal et al., 2017). Additionally, ZnO has been synthesized using various plant extracts such as golden rain tree, prayer beads, chamomile, olive leaf, and tomato (Naseer et al., 2020).

3.7. Titanium dioxide (TiO2) nanoparticles

Titanium dioxide (TiO2) nanoparticles are of interest due to their surface chemistry and morphology, exhibiting antimicrobial, antibacterial, and antifungal properties. Additionally, they show high photocatalytic activity. The biological synthesis of TiO2 nanoparticles, which does not exhibit toxic properties, is more favorable. Numerous plant extracts and applications have been used for synthesis. In a study, butterfly pea flower was utilized, and irregularly shaped particles with sizes ranging from 25 to 110 nm were reported (Goutam et al., 2018).

4. Nanoparticle Characterization Methods

4.1. Microscopy-Based Characterization Techniques

Physical characterization of nanomaterials is carried out through microscopy-based characterization methods. Physical characterization provides information about the structure of nanomaterials. Descriptive parameters such as particle size, particle size distribution, shape, morphology, and surface area are investigated as part of the physical characterization process (Holbrook et al., 2015).

4.2. Optical Microscopes

The optical microscope, often referred to as the light microscope, is the oldest design among microscope types. It is commonly used to magnify and examine the topography of objects that are not visible to the naked eye, utilizing a lens system and visible light (Di Gianfrancesco, 2017; Ebnesajjad, 2014; Rodríguez & Ji, 2018). Optical microscopes have three main functions: magnifying the examined samples, improving the resolution of the magnified image, and adjusting the contrast of the image (Abramowitz, 2003). Unlike other methods, optical microscopes offer the advantage of not being dependent on environmental conditions such as a vacuum. However, they lag behind other methods in terms of resolution. The resolution of optical microscopes is limited by the wavelength of visible light (Sinha Ray, 2013) and is based on the principle of diffraction.

4.3. Elektron Mikroskopları

From the very beginning, the goal of the electron microscope has been the imaging of solid specimens, and techniques for preparing thin samples were discovered later (McMullan, 1995). The electron microscope (EM) enables the visualization and characterization of nanoscale materials. The significant resolving power of the electron microscope is a result of applying an electron beam with a wavelength much shorter than nanometer scale. As the wavelength of the electron beam is much shorter than that of light, the resolution of the electron microscope is higher. For an electron microscope operating with an acceleration voltage of 100 kV, the wavelength value would be 0.0037 nm. Two main imaging techniques are used for electron microscopy detection and characterization: SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy) (Dudkiewicz et al., 2011).

The electron microscope will continue to evolve in the coming years, providing ultra-fast and multi-dimensional growth. Advances in electron sources, faster detectors, and innovative tools, including specialized spectrometers, will push the electron microscope to the next level over time. Such developments will lead to progress in various fields of science and engineering (Zhu et al., 2015).

4.4. Scanning Electron Microscopy (SEM)

Today, scanning electron microscopy is widely used in various fields of science and technology, and significant advancements have been made in the performance of scanning electron microscopy with the development of X-ray detectors (Reimer, 2000). Scanning Electron Microscopy (SEM) is a primary tool for examining the morphology and chemical characterization of nano-sized structures (Sadik et al., 2014). This technique can be used to

obtain images with a large depth of field where the entire field of the micrograph is in focus for imaging the surface of the sample. It is often used in conjunction with Energy-Dispersive X-ray Spectroscopy (EDX) to determine the characteristics of the compositions and orientations of samples (Raval et al., 2018). It is an important method for analyzing and identifying elements in the sample using EDX to characterize material properties and determine the orientation of compositions (Ebnesajjad, 2014). SEM scans the sample surface with a low-energy electron beam, typically ranging from 1 to 30 keV, detects the scattered electrons, and then creates an image. This technique provides three-dimensional images (Dudkiewicz et al., 2011). Additionally, it has the ability to visualize thin films (Sadik et al., 2014). Besides material characterization, scanning electron microscopy can be used for in-situ material engineering and production. As an alternative to SEM, Field Emission Scanning Electron Microscopy (FESEM) has emerged, which provides much higher-resolution images. However, it requires operation under high vacuum conditions (Ceylan et al., 2020).

In the general structure of the SEM device shown in Figure 4.1, there are components such as the electron optics system, sample stage, secondary electron detector, image display unit, and operating system. The electron optics system consists of an electron gun, condenser lens, objective lens, scanning coil, and other components. The electron optics system and the region surrounding the sample are maintained in a vacuum.

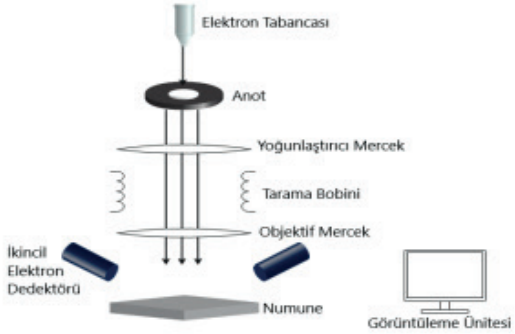

Figure 4.1. *Basic structure of a conventional SEM device.*

4.5. Transmission Electron Microscopy

Transmission electron microscopy (TEM) is one of the most popular instruments among electron microscopes for nanomaterial characterization (Senthil Kumar et al., 2019). TEM has been used for nanomaterial characterization since World War II. TEM provides information about structural details and chemical compositions of materials at various scales, down to the atomic dimensions (Mayeen et al., 2018). Electrons from the source pass through the

sample and lenses to obtain an image on a fluorescent or CCD screen. The schematic representation of a classical TEM device is shown in Figure 4.2.

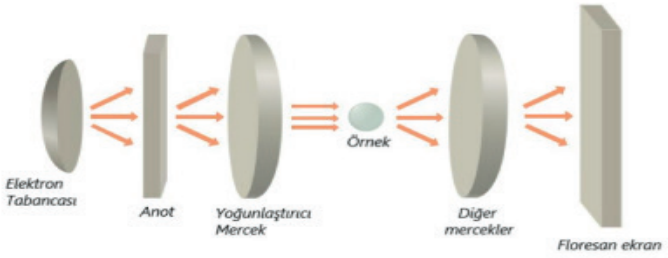

Figure 4.2. *Schematic diagram of a classical TEM.*

4.6. Atomic Force Microscope (AFM)

Atomic Force Microscope (AFM) is one of the microscope techniques used to analyze any solid surface with high resolution and provide information about morphology and surface features (Holbrook et al., 2015). This technique is more advantageous in terms of ease of use, precision, and versatility compared to other surface examination techniques (Moreno Flores, 2009; Toca-Herrera, 2009).

4.7. Scanning Tunneling Microscope

The scanning tunneling microscope (STM) is a member of the family of scanning probe microscopes. It is employed to examine the surfaces of samples at the atomic scale (Ghasempour & Narei, 2018; Lopez-gasso, 2018). STM enables the achievement of a small beam diameter and high current density, while striving to minimize low electron energy and proximity effects. Additionally, STM offers numerous advantages, such as its small size and cost-effectiveness. However, its disadvantage lies in its low operating speed (Griffi, 1990; Kochanski, 1990).

4.8. X-ray Related Characterization Techniques

4.8.1. X-ray Diffraction (XRD)

X-ray Diffraction (XRD) is a technique used for the characterization of crystalline materials. It provides information about parameters such as the structures, grain sizes, phases, crystallinity, strain, and crystal defects of materials (Bunaciu et al., 2015). Additionally, XRD is extensively used in determining the structure of polymer nanocomposites and in studies of process kinetics. The differentiation of shapes and structures of nanomaterials can be investigated by monitoring the intensity, shape, and position of XRD patterns (Turhan, 2010).

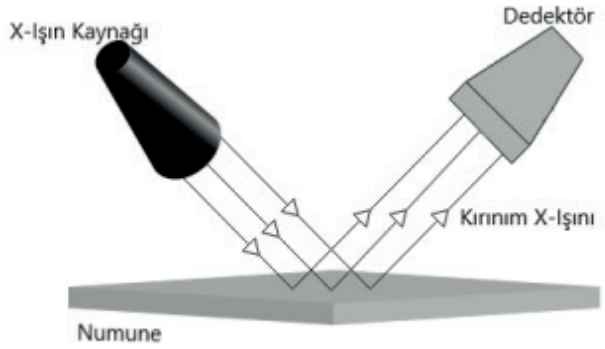

Figure 4.3. *General principle of XRD study.*

Figure 4.3 illustrates the working principle of the XRD device and X-ray diffractions. As shown in the figure, X-rays are sequentially generated in the X-ray tube, filtered to produce monochromatic radiation, focused, and directed towards the sample. X-rays are diffracted, and the diffracted rays are detected, processed, and counted (Bunaciu et al., 2015).

4.8.2. X-Ray Photoelectron Spectroscopy (XPS)

X-ray Photoelectron Spectroscopy (XPS) is an advanced technique that provides chemical information about the surfaces of materials. This technique conducts analysis under vacuum conditions, where the sample is irradiated with X-rays to induce electron emission. The results of the material analysis are determined by the energy difference between the emitted electrons and the X-ray photons (Şavk, 2019). In other words, XPS relies on the photoelectric effect, which involves the emission of electrons from surfaces exposed to sufficiently high-energy light (Grzegorz Greczynski, 2020; Hultman, 2020). XPS is applied to acquire two types of information: the first involves the energy of the photon-excited core electron, which varies depending on the chemical state of the corresponding atom in the surface region. It is commonly used to extract information about the chemical state of atoms in the surface region. The second provides information about the peak intensity, revealing the concentration of corresponding atoms in the surface region and helping to determine the atomic composition (Tougaard, 2021).

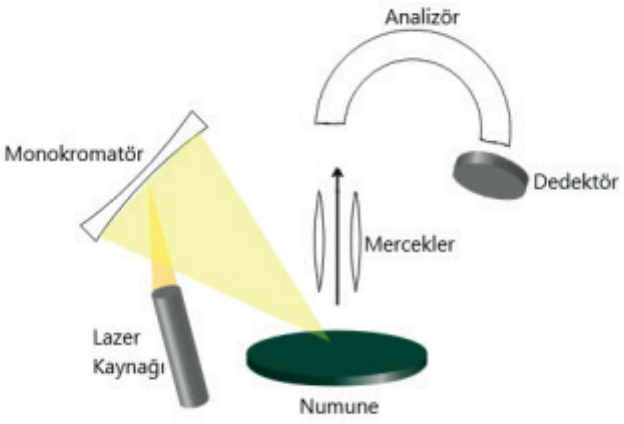

Figure 4.4. *Schematic representation of classical XPS.*

Figure 4.4 provides a schematic representation of a typical laboratory-based XPS instrument with a hemispherical analyzer. X-rays are generated using electron bombardment from a water-cooled anode. The anode emits various X-ray energies, but it peaks due to X-ray fluorescence. The monochromator serves to select a narrow range of X-ray energy around one of these peaks and refocus the X-rays onto the sample. Electrons emitted from the sample pass through a lens, which alters their kinetic energy and focuses them onto the entrance of the analyzer. The entering electrons pass through the analyzer and strike the detector after passing through the exit slit (Shard, 2019).

4.8.3. X-Ray Diffraction Topography (XRT)

The physical properties of the used crystals are directly related to their structural features, making X-ray diffraction techniques indispensable for examining material defects. Among these techniques, X-ray diffraction topography is also included (Lider, 2021). X-ray diffraction topography (XRT) has been used in material characterization with the emergence of synchrotron radiation sources (Stojanoff et al., 1996). Among multi-crystal techniques, the pseudo-plane wave topography method also has a special significance. This method is one of the most sensitive techniques for examining local microstrains in crystals. Due to the difficulty of analyzing the diffraction image in single-crystal XRT, the need for extracting multi-crystals has arisen (Suvorov, 2018). The XRT technique is related to the changes in the directions or intensities of X-rays passing between two points broken by crystals (Bonse et al., 1966). X-ray diffraction topography (XRT) is one of the X-ray diffraction methods that provides information about structural defects in single-crystal materials. While this technique does not provide information about the outer surface of the sample, it shows local variations in distances between planes

and their rotations (Izumi et al., 1996) (Lider, 2021) (Suvorov, 2018).

4.9. Spectroscopy-Based Characterization Techniques

4.9.1. Ultraviolet-Visible (UV-VIS) Spectrophotometer

UV-VIS spectroscopy is a type of absorption spectroscopy in which light in the UV region (200-400 nm) is absorbed by a molecule (Chirayil et al., 2017). The spectrophotometer setup consists of a light source, wavelength selector, detectors, and lenses, as shown in Figure 4.5. The lenses collect, focus, reflect, split into two beams, and send the light onto the sample at a certain intensity (Nazik, 2012).

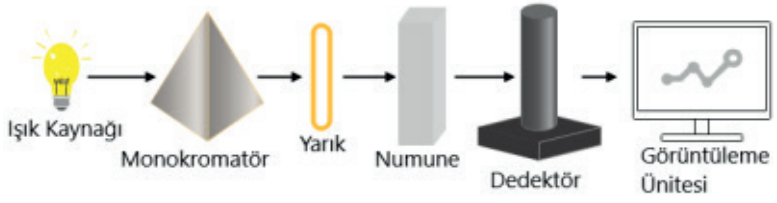

Figure 4.5. *General working principle of a spectrophotometer.*

UV-VIS spectroscopy is used to assess the size, concentration, aggregation degree, and stability of nanoparticles The UV-Vis spectrometer measurement graph of silver nanoparticles synthesized from the *Sideritis brevidens* species at different concentrations in the 200-600 nm absorbance range is provided in Figure 4.6. Additionally, it provides information about electronic transitions occurring in the material. It is a suitable method for predicting the optical band gap by investigating electronic transitions between the valence band and the conduction band in semiconductors (Z. Chen et al., 2013).

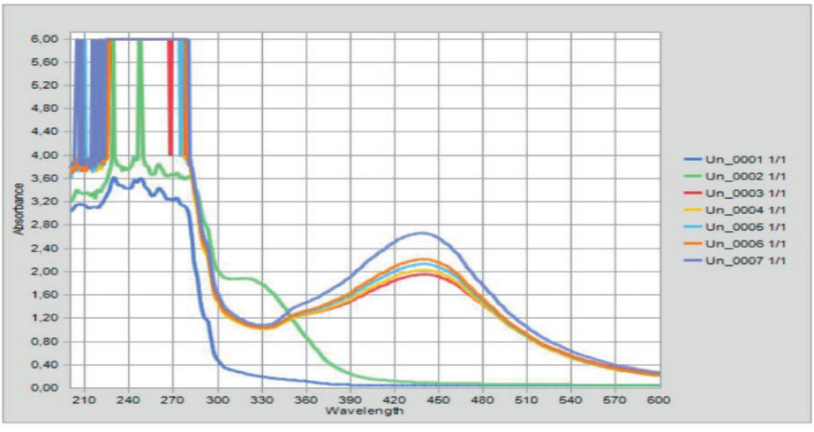

Figure 4.6. *Absorbance graph of AgNPs obtained at different concentrations through green synthesis from Sideritis brevidens in the 200-600 nm wavelength range.*

4.9.2. Fourier Transform Infrared Spektroskopisi (FT-IR)

Fourier Transform Infrared Spectroscopy (FTIR) is used for the identification of organic, inorganic, and polymeric samples by utilizing infrared light. It is a spectroscopic technique that provides the infrared spectrum of absorption and emission of samples. Changes in characteristic absorption bands indicate alterations in material composition (Dwivedi et al., 2017; Titus et al., 2019). The primary goal of FTIR spectroscopy is to measure how much light of different wavelengths the samples absorb. Sample pellets are prepared with potassium bromide (KBr) salts due to their transparency, ensuring better spectrum resolution (Rakhee et al., 2018).

FTIR spectroscopy provides information about the distribution of elements and molecules in samples. In FTIR spectroscopy, radiation from the source passes through the interferometer and then through the sample before being detected by a detector. The signal is converted to a digital signal and transferred to the computer. Fourier transformation is performed with the computer (Titus et al., 2019). The schematic diagram of FTIR spectroscopy is shown in Figure 4.7.

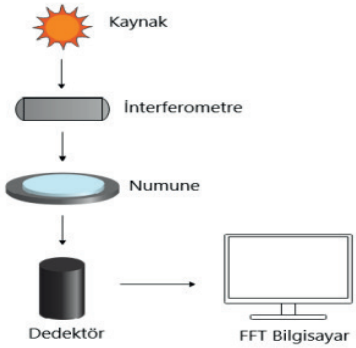

Figure 4.7. *Schematic diagram of a classical FT-IR system.*

CONCLUSION

The conversion of renewable agricultural materials or food waste into energy and useful by-products is an environmentally focused research area that can be significantly enhanced with nanotechnology (Adeola et al., 2019). Plants, in general, have a low environmental impact while producing organic components used in biological synthesis. They are more environmentally friendly and economically advantageous compared to chemical synthesis methods.

Green synthesis conducted using *Sideritis* species is a significant step in environmentally friendly metal nanoparticle production. This process has a lesser environmental impact than chemical methods. Additionally, the biolog-

ical activities of the obtained metal nanoparticles enable various applications in biotechnology. These activities can range from antimicrobial properties to biomedical applications. When combined with environmental sustainability, these methods can provide sustainable and effective solutions in the fields of the environment and biotechnology. The convergence of environmentally friendly production methods with a wide range of biological activities can lead to significant advancements in biotechnology.

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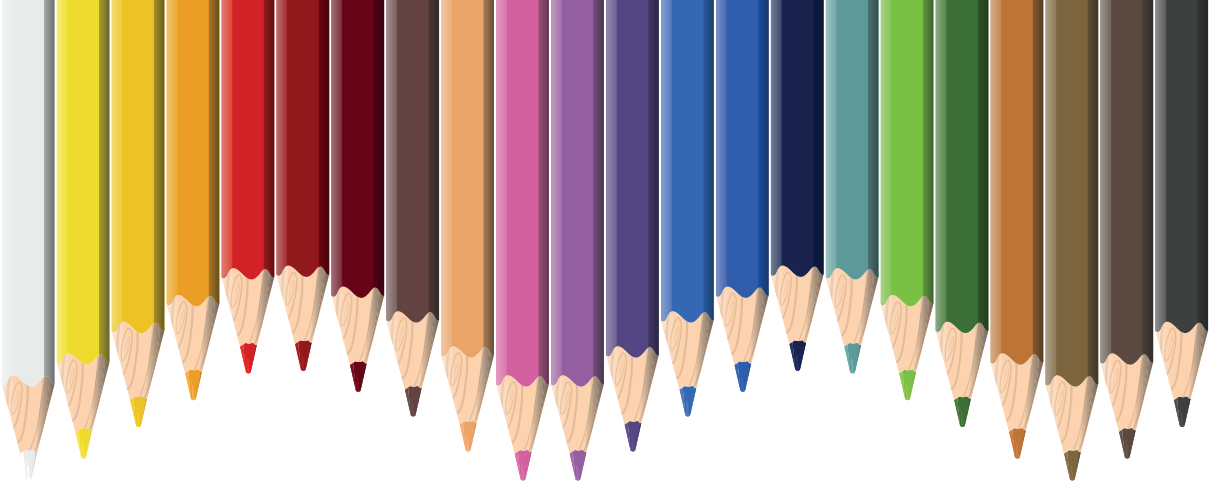

Chapter 4

A MICROBIOLOGY RESEARCH ON HALLOUMI CHEESE PRODUCED IN NORTH CYPRUS

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*This study was obtained from the master's thesis completed in 2016. Prepared by: İhsan Erol Özçil, Thesis Advisor: Assoc.Prof.Dr. Serdar Susever, Thesis Title: A Research on Occurrence of Salmonella and Staphylococcus Aureus in Halloumi Cheese Produced in Turkish Republic of Northern Cyprus.

1.Introduction

An unripened semi hard and brined cheese, known especially as Halloumi / Hellim as a Cypriot brand is produced generally through a mixture of milk of cow or sometimes the goat and sheep (Ayto, 1990). Due to its special texture formed thanks to its high melting point, this cheese can either be grilled or fried. Halloumi is differentiated to other kind of white cheese with the preparation method that includes rennin, consisting either no acid or bacterium that produces acid (Ayto, 1990; Gibbs et al., 2004). Halloumi cheese is the traditional cheese of Cyprus, holding the traditional title of Cyprus, Halloumi is also consumed widely in countries like Jordan, Lebanon and also Turkey. Originally found during the Medieval Byzantine era (AD 395-1191) then later became a popular product within the Middle East. On April 12, 2021, the European Commission announced that it has adopted an important package for the protection of the Halloumi product in the European Union, which is currently protected as a name of origin in both the Turkish Republic of Northern Cyprus and the Republic of Turkey on behalf of the Turkish Cypriot Chamber of Industry (European Commission, 2021). For this reason, the value of halloumi cheese has reached high levels internationally. Salmonella spp. is a non-sporing rods, gram negative, catalase negative and oxidase negative (Quinlan et al., 2000). It is a pathogen with one of the most repetitive frequency rate in the industry of food. Being one of the mostly encountered base for the foodborne diseases, especially in dairy products, the historical roots to this microorganism reaches a century. Accordingly most of the species found within Salmonella spp. are pathogenic (Kasrazadeh & Genigeorgis, 1994; De W Blackburn & McClure, 2009). Annual cases for typhoid fever reaches up to 16 million as gastroenteritis cases reaches up to 1.3 billion and finally Salmonella death rate in the world reached up to 3 million deaths (Bhunia, 2008). In short, Salmonella can be described as a bacterium sized approximately 2-3 x 0.4-0.6 μm and which is rod shaped gram negative flagrated facultative anaerobe (Montville & Matthews, 2008). S. aureus an oxidase negative, catalase positive and Gram-positive cocci. It forms clusters showing pigmented colonies when grown in nutrient agar. Clumps, rather characteristic, can be seen bunches of grapes (Quinlan et al., 2000). Staphylococcal food poisoning also known as Staphylococcal intoxication is due to the ingestion of enterotoxins formed when S. aureus growth in foods. Human origin consists of Enterotoxin production as the most common S. Aureus which holds sound correlation to the enzyme coagulase production (Sutherland & Varnam, 2002).

2. MATERIAL AND METHOD

2.1 Halloumi Sampling for Salmonella

Halloumi cheese samples collected from different markets in TRNC were used as material in this study. Halloumi cheese samples have been gathered randomly specifically from the business sector stands, transported to lab environment by means of unbroken cool chain.

2.1.2 Media and Test Kids Utilized For The Isolation and Identification of Bacteria

Peptone Water (BPW, Oxoid CM 0509) It has been utilized for dissolved during theseparation stage.

Segments

- In sterile stomacher packs 500's
- Buffered Peptone Water 225 ml
- pH 7.2±0.2

25 gr of sample was weighed and homogenized with 225 ml of pH-adjusted pepton water(BPW, Oxoid CM 0509), then transferred into stomacher bag and incubated at 37 °C for 24 hours.

Mc Conkey Agar

- It is the particular medium utilized for the identification of *Salmonella*.

Segments

- Rappaport-Vassiliadis Soya Peptone Broth (RVS, Oxoid CM 0866) 10 ml
- Muller-Kauffmann Tetrathionate/novabiocin Broth (MKTTn, Oxoid CM 1048) 10 ml

2.1.3 Strategy of Salmonella

25 gr of cheese sample was homogenized with 225 ml of sterilized Buffered Peptone Water (BPW, Oxoid CM 0509) for 1 minute and then incubated at 37 °C for 24 hours in a stomacher bag. This 0.1 ml in 10 ml of pre-enrichment medium fluid Rappaport-Vassiliadis soya peptone juices (RVS, Oxoid CM 0866) to tubes containing 1 ml in 10 ml of Muller-Kauffmann tetrathionate/ novabioc Broth (MKTTn, Oxoid CM 1048) which will be immunized into tubes. RVS tube at 42°C, while MKTTn tubes in the wake of being brooded for 24 hours at 37 °C after hatching time and MacConkey Agar from every

tube and Salmonella-Shigella (SS) agar exchanging on loaded with a circle with a round-finished circle, 37 °C, will be left for 24 hours brooding. Then MacConkey agar yellow in shading and dark in light of SS agar, Salmonella suspect states will be recognized utilizing the BD Phoenix gadget.

Evaluation of Test Results

- It was reasoned that from the halloumi tests, no decided defilement rate was seen Salmonella.

2.2 Halloumi Sampling of S. Aureus

In this study, 10 halloumi test samples collected from the market sectors of TRNC on different days were used as material. Halloumi cheese samples have been taken randomly straightforward from the business sector stands, transported to research laboratory environment by means of unbroken cold chain and have been broke down inside a brief span for S.aureus presence.

1.1.1 Media and Test Kids Utilized For The Isolation and Identification of Bacteria

Peptone Water (Oxoid CM0009) It has been utilized for dissolved during the detachment stage.

Segments

- Peptone 10.0 g
- Sodium Chloride 5g
- Distilled Water 1000 ml
- pH 7.2±0.2

15 g of prepared mix from the medium has been dissolved in 1000 mL refined water and setat pH 7.2±0.2. The readied medium base has been appropriated to 9 mL tubes (second dissolved) and 90 mL bottles (first dilution) and has been left to cool after auoto-clave cleansing at 121°C for 15 minutes.

Baird-Parker (BPA) Agar Base (Oxoid CM0275)

- It is the specific medium utilized for the ID of Staphylococcus aureus. (FDA/BAM, 2001).

Segments

- Tryptone 10.0 g
- Lab-Lemco'powder 5.0 g

- Yeast Extract 1.0 g
- Sodium Pyruvate 10.0 g
- Glycine 12.0 g
- Lithium Chloride 5.0 g
- Agar 20.0 g
- Distilled Water 1000 ml
- pH 6.8±0.2

Egg Yolk-Tellurite Emulsion (OXOID SR0054 C)

- 50 ml Egg Yolk Tellurite Emulsion contains approximately 3 ml 3.5% potassium tellurite.

Staphytect Plus (OXOID DR 0850) Latex Agglutination Test Kid Parts

- DR851M Staphytect Plus Test Reageny (5,6 ml)

Blue latex particles covered with both porcine, fibrinogen and rabbit IgG together with particular polyclonal antibodies raised against capsular polysaccharide of *S.aureus*. Every container contains adequate reagent for 100 tests. DR852M Staphytect in addition to Control Reagent (5,6 ml) Blue unsensitised latex particles. Every container contains adequate reagents for 100 tests. DR500G Reaction Cards. There are 35 expendable response cards.

2.2.3 Strategy of S. Aureus

Initial Step: Bring the latex reagents to room temperature. Ensure that the latex reagent is blended by incredible shaking and remove any latex from the dropper pipette for complete blending. Dispense 1 drop of test latex onto one of the circles on the response card and 1 drop of control latex onto another circle. Using a circle, get and spread what might as well be called 5 normal measured suspect *Staphylococcal* provinces (identical to 2-3 mm breadth of development) onto a circle from a society media plate and blend this in the Control Latex reagent. Spread to cover the circle. Dispose of the circle properly. Using a different circle continue similarly with the Test Latex. Pick up and shake the card for up to 20 seconds and watch for agglutination under typical lighting conditions. Try not to utilize an amplifying glass.

Evaluation of Latex Test

An outcome is sure if agglutination of the blue test latex particles happens and a smooth blue suspension stays following 20 seconds. This hypothetically

distinguishes the strain as *S.aureus*.

A negative result is acquired if no agglutination happens and a smooth blue suspension stays following 20 seconds in the test circle. This possibly distinguishes the strain as non *S.aureus*.

Slight graininess of the test latex joined by no adjustment in the presence of the control latex ought to be translated as an obscure result. Strains ought to be re-tried after subculture onto non-specific media. The test is uninterpretable if the control reagent demonstrates agglutination. This shows the way of life causes auto agglutination.

Constraint of The Procedure

The propensity of detached settlements to bring about auto agglutination increments withbrooding times past the prescribed 36 hours duration.

The immune response utilized as a part of Staphytect Plus has been upgraded to dodgepotential cross-responses with shared antigens from coagulase negative *staphylococci*.

Planning

63 g prepared blend from the BPA medium has been dissolved in 1000 mL refined water, set at pH 6.8±0.2 and totally broke down in high temp water shower. After autoclave disinfection at 121°C for 15 minutes, it has been cooled to 50°C and after that 50 mL Egg-Yolk Telluride Emulsion (Oxoid SR 0054, it contains potassium tellurite and is in ready100 mL fluidstructure) has been included and blended. The readied arrangement is poured onto clean petri dishes and left to cool at room temperature.

2.3 Isolation and Identification of S.aureus

2.3.1 Cultivation in Strong Medium Base and Assessment of Settlements

The distinguishing proof of coagulase positive *staphylococci* by exemplary system has been done as per FDA/BAM (2001). 10 g test has been taken in a way to speak to the entire of the specimen item and put in stomacher pack. At that point, 90 mL peptone water has been included and homogenized in stomacher for 60 seconds. 1 mL arrangement was taken by pipette from the homogenate (10 - 1) and similarly conveyed and developed on pre-arranged BPA medium plates by spread plate system. The petri dishes were, then, left for hatching in anaerobe environment at 35 ±1°C'd for 24-48 hours. The same strategy and technique has been rehashed for each of the 34 tests. Toward the

end of the hatching time frame, the sparkling dark dim settlements with a measurement of 2-3 mm and hovered by limited, curved molded, smooth, gleaming zones were considered as plausible S.aureus states and checked.

Confirmation

Latex affirmation test was connected to likely S.aureus strains which multiplied as an results of BPA medium development. Latex reagent has been conveyed to room temperature and homogenized by shaking. A short time later, one drop of test reagent has been poured on each test curls on the response card; five suspicious settlements were taken from the petri dish with sterile circle; and were spread over the test loop whereby they blended with the test reagent. The response card has been shaken in roundabout developments for 20 seconds and watched for agglutination.

As agglutination was seen by the testing of all settlements, the outcome was viewed as positive. We continued to the second period of affirmation and rehashed all the previously mentioned stages with a control reagent.

The testing of the investigated colonies brought about agglutination with latex test reagent, however no agglutination was seen with the control reagent. It was, hence, affirmed that the confined S.aureus states were, truth be told, coagulase positive strains.

3.RESULT

3.1 Result of Salmonella Analysis

A total of 10 halloumi cheese samples were collected from the markets and analyzed for Salmonella. Salmonella was not detected in any of the 10 samples Table 1.

Table 1: Positive Test Results for Interested Microorganisms in Halloumi Samples by BD Phoenix Device

Label Sequence No.	S.aureus kob[(iv)]/g	E.coli kob[(iv)]/g	Coliform Bacteria kob[(iv)]/g	Yeast and Mold kob[(iv)]/g	Salmonella spp. (25 g.)	Listeria monocytogenes (25 g.)	E.coli 0157(25 g.)
	Limit[(i)]:0/g Limit:[(iii)]:10^3/g	Limit[(i)]:0/g	Limit:[(i)]:10^3/g	Limit:[(i)]:10^3/g	Limit:[(i)(ii)]: 0/25g	Limit:[(ii)]:0/25g	Limit:[(iii)]:0/25g
1	0	0	0	0	Not Detected	Not Detected	Not Detected
2	0	93	93	$5x10^2$	Not Detected	Not Detected	Not Detected
3	0	**≥2.4x10^3**	**≥2.4x10^3**	$1x10^2$	Not Detected	Not Detected	Not Detected
4	**7x10^3**	**≥2.4x10^3**	**1100**	**5x10^4**	Not Detected	Not Detected	Not Detected
5	0	0	0	$4.5x10^2$	Not Detected	Not Detected	Not Detected

6	0	0	0	0	Not Detected	Not Detected	Not Detected
7	0	0	0	0	Not Detected	Not Detected	Not Detected
8	0	0	0	0	Not Detected	Not Detected	Not Detected
9	0	0	0	0	Not Detected	Not Detected	Not Detected
10	0	0	0	0	Not Detected	Not Detected	Not Detected

3.2 Result of S.aureus Analysis

As a result of the analysis of 10 halloumi samples collected from different markets in TRNC, S.aureus was detected in 2 out of 10 samples (6%). Figure 1. It was within the accepted limits cited in the Turkish Food Codex Regulation on Microbiological Criteria and did not endanger public health.

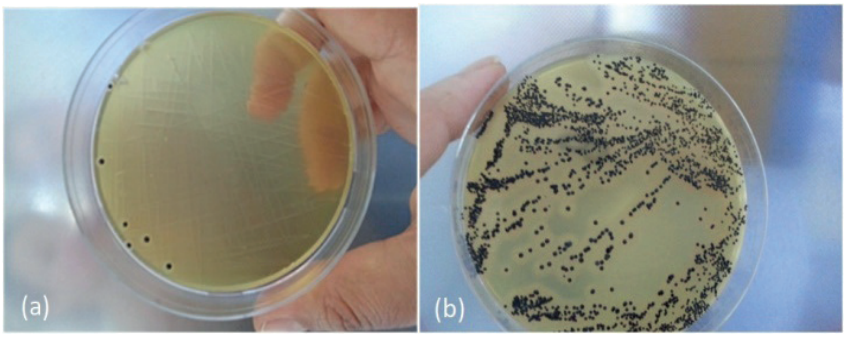

Figure 1: *S.aureus colonies isolated from Halloumi Cheese sample. (a) Below limit, (b) Above limit*

3.3 Confirmation

All the suspected colonies from Baird Parker Agar (BPA) were tested by Oxoid Staphytect Plus type Latex test for confirmation. The results of the latex agglutination test are summarised in Table 2 according to sample distributions. Two Halloumi samples containing S. aureus after BDP test were confirmed as S. aureus with Latex Agglutination Test.

Table 2: Positively Confirmed Test Results by Latex Agglutination Test				
Label Sequence No.	**Local**	**Area**	**S.aureus Latex Test Result**	**S.aureus Colony Count (CFU/ml)**
1	+	Lefkoşa	Positive	$<1.10^3$
2	+	Lefkoşa	Positive	$\leq 1.10^3$
3	+	Girne	Negative	Negative
4	+	Girne	Negative	Negative
5	+	Güzelyurt	Negative	Negative
6	+	Güzelyurt	Negative	Negative
7	+	İskele	Negative	Negative
8	+	İskele	Negative	Negative
9	+	Gazimağusa	Negative	Negative
10	+	Gazimağusa	Negative	Negative

4.DISCUSSION AND CONCLUSION

In a study carried out in 2009 at Kütahya Public Health Laboratory, nose cultures were controlled over a time of right around one year (May 2006-June 2007) from a total of 3048 individuals. S.aureus colonies were detected from 217 examples. 37 (17.05 %) of these were originated from tests of female contributors and 180 (82.9%) originated from male contributors (Gülbandılar, 2009). In a study found that S.aureus presence in one sample out of 10 milk test samples tested in India and reported the S.aureus count as $4.5x10^1$ CFU/g (Önganer & Kirbag, 2009). In a study completed a microbiological study in Diyarbakır with 30 Cokelek Cheese samples. They identified S.aureus in the majority of the specimens and reported the least S.aureus count as $6x10^6$ CFU/g and the most noteworthy $10.28x10^6$ CFU/g (11). In a joint study of studied 125 examples of vacuum-packed cheddar cheese sold in Bursa. Thus, they found S.aureus presence in four samples and reported most astounding S.aureus count as $1.8x10_3$ CFU/g and least S.aureus count as $1.0x10^2$ CFU/g (Günşen & Büyükyörük, 2003). In a study conducted in Bursa, found coagulase (+) staphylococcus in crude milk samples and decided that the microbes count as $1.9x10^4$ CFU/mL, recognized staphylococcus/micrococcus colonies in pasteurized milk samples and decided that the microorganisms count as $103\text{-}10^4$ CFU/mL, and decided coagulase (+) staphylococcus count in salt water $5.0x10^4$ CFU/g (Evrensel, 2003). A total of 10 samples of halloumi cheese produced in different regions were collected from various markets of the island. Whereas no Salmonella was observed in any sample, 2 out of 10 samples were containing Staphylococcus aureus (S.aureus). However these samples of S. aureus colonies were lower than risk levels according to Turkish Food Codex. The frequency of Salmonellosis and S. aureus should not be disregarded because of the staggering impacts on human health. Awareness about Salmonella and S. aureus its development is vital to ensure food safety.

Intercession methodologies are consequently essential to control Salmonella and S. aureus from farm to fork. Increasing food safety problems worldwide in recent years have increased consumers' awareness of food safety and caused the public to remain distrustful of the increasingly complex and globalized food production and trade system. The frequency of salmonellosis should not be ignored due to its surprising effects on human health. Awareness about Salmonella and its development is vital to guarantee food safety and security. Consequently, intercession methodologies are essential to control Salmonella and Staphylococcus aureus from farm to table. A total of 10 samples of halloumi cheese produced in different regions were collected from various markets in Nicosia (Nicosia). While no samples showed Salmonella, 2 out of 10 samples had Staphylococcus aureus. However, these S. aureus colony samples were below the risk level according to the Turkish Food Codex. We conclude that the contamination levels in the samples are below the minimum infectious dose according to the Turkish Food Codex Microbiological Criteria Committee and may not pose any danger to public health. The results of halloumi samples obtained from various market sectors of TRNC were examined. It showed that halloumi cheeses were quite clean in terms of Salmonella. However, the use of quality raw materials and basic production techniques are not sufficient to ensure the microbiological quality of dairy products. The possibility of microbiological contamination is high from air dust, apparatus and equipment, personnel, and different sources. Post-sanitization contamination is of particular importance for product quality and the health and well-being of customers. Good quality of crude material satisfactory production methods and other accompanying criteria are critical so as to assure and sustain the bacterial quality of the dairy products in the TRNC and for the general well-being of the public. Our recommendation for these purposes are as follows: Processing of the raw material day ought to be completed at the same, Personnel ought to have routine medical checks, Personnel ought to sufficiently be prepared for microbiological contaminations, Personnel ought to be trained on individual hygiene, Temperature which are of specific significance for microbial development at storage and production sites ought to be entirely controlled particularly in hot summer days in the TRNC, Necessary legislative warning and actions ought to be taken for every single culpable condition. Organizations and producers ought to be educated about good hygiene practices that deliver this sort of food.

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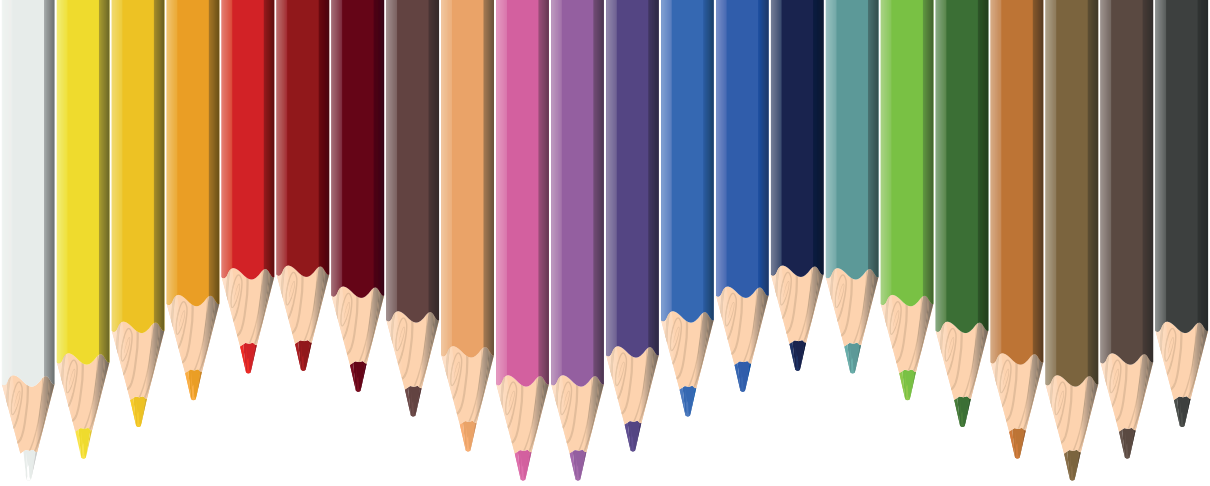

Chapter 5

RECENT ADVANCES ON SUPPORT MATERIALS FOR PORCINE PANCREATIC LIPASE IMMOBILIZATION

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RECENT ADVANCES on SUPPORT MATERIALS for *PORCINE PANCREATIC* LIPASE IMMOBILIZATION

1. Enzymes

Enzymes are extremely versatile natural biocatalysts that are essential to all vital processes, including cell division, metabolism, and immune system reactions. They show high catalytic activity and specificity, are highly effective at catalyzing chemical reactions in particular, and are enantio- and stereoselective (Maghraby et al., 2023; Ozyilmaz and Caglar, 2023; Shanmugasundaram and Pandit, 2022; de Souza Vandenberghe et al., 2020; Fernandez-Lafuente, 2009; Gröger and Hummel, 2014; Li et al., 2021; Ozyilmaz et al., 2023; Sampaio et al., 2022; de Souza Vandenberghe et al., 2020; Bilal et al., 2019; Wei et al., 2019; Yilmaz, 2012). They are vital in regulating basic functions in metabolic processes (de Souza Vandenberghe et al., 2020).

Enzymes are regarded as significant "green tools" due to their unique properties and broad range of applications, according to research (Bijoy et al., 2022). In addition to many fields around the world, from biotechnology, health, paper and pharmaceutical industry to bioprocess, food and textile industry, they also play important roles in medical diagnoses (de Souza Vandenberghe et al., 2020). Enzymes have long been employed in many different industrial sectors to simplify intricate production steps and procedures. Today, the demand for sustainable processes is greatly increasing the application of enzymes as catalysts in various industrial sectors. (Maghraby et al., 2023; Ranjbari et al., 2019). Enzymes are essential components of many industrial products (Maghraby et al., 2023). Although they have been used in various industrial sectors for a long time, there has been a continuous increase in their use in industrial sectors, especially in the last twenty years (Maghraby et al., 2023).

There are different subclasses of enzymes (de Souza Vandenberghe et al., 2020). Hydrolases are a prominent class in industrial applications due to their low cost, high specificity, and ease of access. Amylases, proteases and lipases are enzymes that play an important role in industrial application and are among the most prominent enzymes in the industry (Filho et al., 2019; Klinman and Hammes-Schiffer 2013; Homaei, 2015; Pera et al., 2015). As the most well-known hydrolase enzymes, lipases are the most frequently used enzymes in biocatalysis and have enormous industrial potential because of their versatility in catalyzing both synthetic and hydrolytic processes (Ozyilmaz et al., 2023; Sampaio et al., 2022; Salgado et al., 2022; Pereira et al., 2022; Tarczykowska, 2018; de Araújo et al., 2017; Dhake et al., 2013). They play an important role in numerous metabolic reactions in all living species (Zaitsev et al., 2019; Zaitsev et al., 2010; Sharma et al., 2001; Vulfson, 1994).

Lipases can be obtained from all living species, from animals, plants or microorganisms. It is preferred to use lipases derived from microorganisms rather than those derived from plants or animals in industrial applications (Sampaio et al., 2022). Lipases have an active site formed by a catalytic triad, usually consisting of histidine, serine, aspartic or glutamic acid (Sampaio et al., 2022; Ozyilmaz et al., 2021; Kapoor and Gupta, 2012). Most lipases also have a polypeptide chain covering their active site, called a lid (Sampaio et al. 2022; Grochulski et al., 1994; Grochulski et al., 1993; Brzozowski, et al., 1991; Winkler et al., 1990). Lipases exhibit exceptional catalytic activity in a variety of reactions along with good stability. They can also catalyze a wide range of reactions, including acidolysis, esterification, and transesterification, thanks to their high specificity (Ozyilmaz et al., 2022; Sampaio et al., 2022; Akil et al., 2020; Abed et al., 2017; Facin et al., 2019; Stergiou et al., 2013; Hari Krishna and Karanth, 2002). They do not require cofactors, can generally be used over wide pH and temperature ranges, recognize a wide range of substrates but have high specificity, regio- and enantio-selective abilities (Maghraby et al., 2023, Sampaio et al., 2022; Rodrigues et al., 2019; Kapoor and Gupta, 2012 Stránský et al., 2007).

Lipases are utilized to produce goods in a variety of industrial sectors, including biosensors, detergents, food, paper, medicines, textiles, cellulose, biopolymers, cosmetics, and medical diagnostic kits. This is due to their exceptional performance and distinctive characteristics. (Sampaio et al., 2022; Facin et al., 2019; Filho ve ark., 2019; Borowiecki et al., 2017; Dumorné et al., 2017; Hasan et al., 2006; Houde et al., 2004; Sharma et al., 2001).

Among lipases of animal origin, *porcine pancreatic* lipase (PPL) has been among the popular research topics in recent years (Gonçalves, et al., 2021; Rial et al., 2020; Chen et al., 2020; Silva et al., 2014). One of the significant lipolytic enzymes of the intestine that actively promotes the breakdown and absorption of dietary triglycerides and cholesteryl esters is *porcine pancreatic* lipase (PPL) (Vulichi et al. 2023). PPL is a single polypeptide chain of 50 kDa (Hermoso et al., 1996). The typical conserved catalytic triad is formed by Ser-152, Her-263, and Asp-176 out of a single chain comprising 449 amino acids (Vulichi et al., 2023; Liu et al., 2020; De Caro et al., 1981).

Among the numerous commercial lipases available, crude PPL is a notable candidate for industrial applications, with its low cost relative to other types, accessibility, high stability, and broad specificity for biotransformation of non-natural substrates (Gonçalves, et al., 2021; Suo et al., 2019; Jin et al., 2018; Mendes et al., 2012).

The catalytic site of PPL is surrounded by multiple α-helix that help to stabilize and sustain the activity of the enzyme. Among the secondary structural

components of the enzyme, the loops have a very flexible structure. Interaction with the substrate is the determining factor whether the enzyme activity increases or decreases (Zhang et al. 2019).

2. Enzyme Immobilization

Current demands for sustainable and environmentally friendly industrial processes are encouraging the research of new methodologies based on enzyme technology (Sheldon and Brady, 2022; Sampaio et al., 2022; Woodley, 2020; Sheldon and Brady, 2019; Hassan et al., 2019; Sheldon and Woodley, 2018; Ferreira-Leitão et al., 2017). The need for cost-effective, environmentally friendly new methods to preserve the conformational structures of enzymes under harsh conditions and ensure their reusability is increasing day by day (Sheldon and Brady, 2022). Enzymatic immobilization has been proposed as an alternative to reduce the limitations of soluble enzymes, increase their stability against harsh conditions, enable their recovery and reuse, and significantly reduce the cost of processes involving the use of enzymes (Filho et al., 2019; Barbosa et al., 2014; Cao et al., 2003). Immobilization of enzymes provides greater stability to the structure, making it resistant to more severe reaction conditions (Hernandez and Fernandez-Lafuente 2011; Liu et al., 2018; Nishida et al., 2018).

Different enzyme immobilization techniques are used according to the interactions between enzymes and support material (Fig.1) (Liu et al., 2018). The most studied enzyme immobilization techniques include physical adsorption, encapsulation, covalent attachment, cross-linking and entrapment methods (Liu et al., 2018).

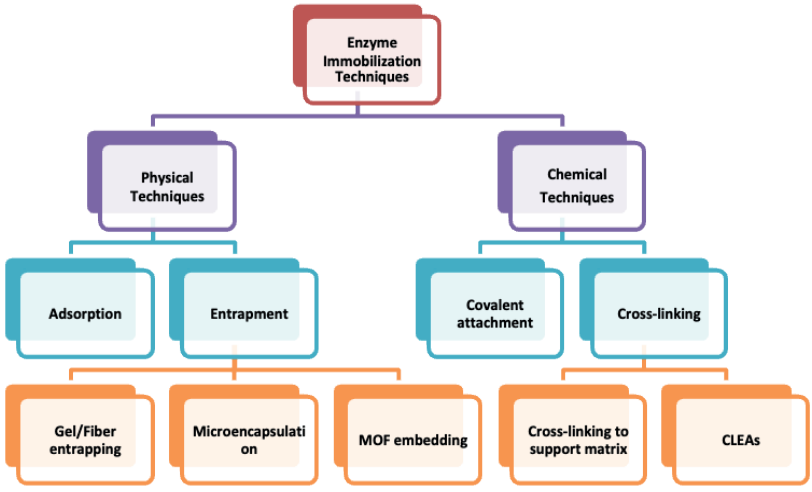


Fig1. *Enzyme immobilization techniques (Liu et al., 2018).*

It is also essential to examine the support material used in the immobilization of enzymes. Enzyme immobilization further increases the use and efficiency of enzymes by improving their stability, reusability and recyclability (Maghraby et al., 2023; Bijoy et al., 2022).

Lipases are enzymes that have the potential to a lot of attention in the industrial field with their properties. However, high purchasing cost, low stability, difficulties in recovery and reuse are important factors that limit their usage areas (Ozyilmaz et al., 2022; Filho et al., 2019; Cao et al., 2003; Barbosa et al., 2014).

Enzyme immobilization is a technique designed to make process control easier and solve the issue of enzyme recovery and reuse (Sampaio et al., 2022; DiCosimo et al., 2013; Liese and Hilterhaus; 2013). Additionally, by using enzyme immobilization, the selectivity, specificity and activity of enzymes can be adjusted, enzyme inhibitions can be reduced or enzyme purification can be achieved (Sampaio et al., 2022; Remonatto et al., 2022; Almeida et al., 2022; Arana-Peña et al., 2021; Adlercreutz, 2013; Garcia-Galan et al., 2011; Mateo et al., 2007; Iyer and Ananthanarayan 2008).

In the research conducted so far, lipases have been immobilized with different protocols using different supports (Sampaio et al., 2022; Ozyilmaz et al., 2019; Filho et al., 2019).

Porcine pancreatic lipase (PPL) is widely used in many industrial fields due to its high enantioselectivity in biocatalysis. (Chen et al., 2020; Li et al., 2017). However, lipases' poor stability and difficulty in reusing make them limited in a wide range of industrial applications. To overcome these disadvantages, increase enzyme stability and create a robust biocatalyst, the application of enzyme immobilization techniques comes to the fore (Ozyilmaz et al., 2023; Sampaio et al., 2022; Bilal et al., 2019). It is important to select the appropriate support material to maintain the activity and stability of the lipase structure under harsh conditions (Ozyilmaz and Caglar, 2023; Boudrant et al., 2020).

Various materials can be used as supports for enzyme immobilization. These materials are generally organic materials, inorganic materials, magnetic nanoparticles, hybrid materials or composite materials. (Ozyilmaz et al., 2021; Suo et al., 2019).

In the research conducted so far, it has been observed that the structure, surface chemical composition and properties of the supports greatly affect the activity and stability of the enzymes during the enzyme immobilization process. Perfect support materials for enzyme immobilization must have certain properties. (Fig 2) (Suo et al., 2019; Eş et al., 2015).

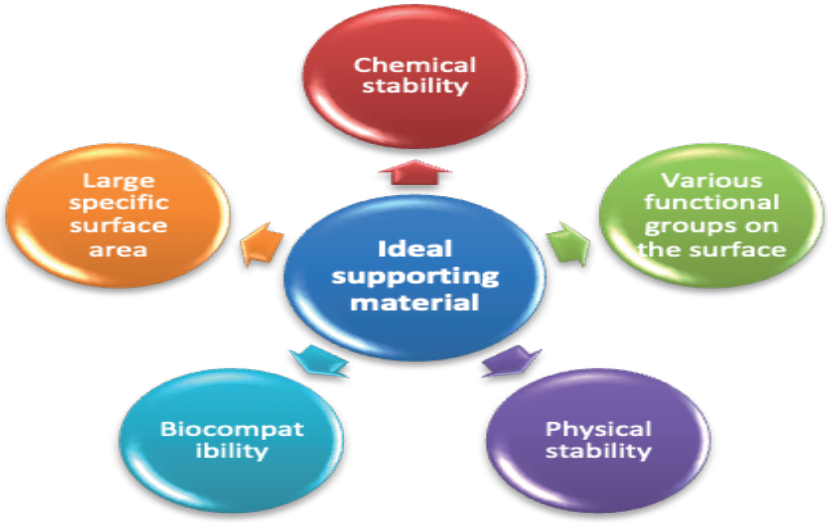


Fig 2. *Ideal supporting material species (Suo et al., 2019; Eş et al., 2015).*

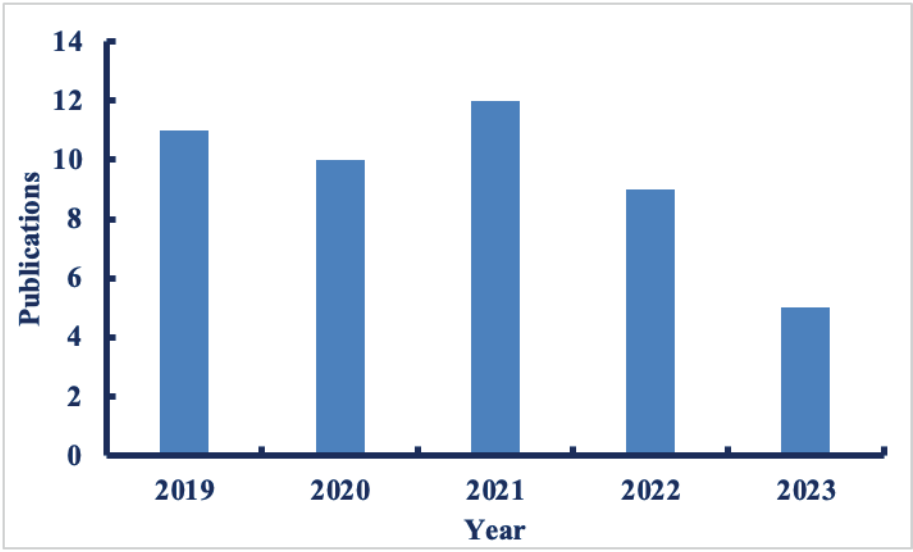
The development of suitable carrier materials can greatly affect the performance of biocatalysts, including hydrophobic carriers in lipase immobilization, which can increase the activity of biocatalysts (Suo et al., 2019; Jin et al., 2018).

Studies on Enzyme Immobilization of PPL in the Last 5 Years

A joint search of "*Porcine pancreatic* lipase" and "enzyme immobilization" between 2019 and 2023 turned up 47 studies in the Web of Science database, according to research done in the last five years. (fig.3). It was observed that 37 of these studies were research articles and 10 were compilation studies.

It was determined that there were 44 studies in the Elsevier database as a result of the joint search of "*Porcine pancreatic* lipase" and "enzyme immobilization" between 2019 and 2023.

The table 1was arranged according to studies conducted on the use of *porcine pancreatic* lipase in enzyme immobilization.



Tablo 1. *PPL immobilization studies in the Last 5 Years*

Support Material	**İmmobilized PPL**	**Immobilization Method**	**References**
IL-MCS	PPL-IL-MCS and GO/PPL-IL-MCS	Adsorption	(Suo et al., 2019)
IL-CS-$Fe_{3O}4$	PPL-IL-CS-Fe_3O_4 and PPL-CS-Fe_3O_4	Surface modification	(Suo et al., 2019)
IL and MSA	PPL-IL-MSA and PPL-MSA	Covalent Binding	(Suo et al., 2019)
PSS and Chitosan	LPP-Chi and LPP-PSS	Adsorption	(Zaitsev et al., 2019)
UiO-66-NH_2	PPL@MOF	Cross-linking	(Chen et al., 2020)
IL-MCMC	PPL-IL-MCMC and PPL-MCMC	Covalent Binding	(Suo et al., 2020)
SBA@CS	SBA@CS-Co-PPL	physical adsorption, cross-linking	(Qiu et al., 2020)
mDE-APTES	PPL@mDE	Covalent Binding	(Vakili et al., 2020)

Nano-SiO_2	Immobilized porcine pancreatic lipase	Surface modification	(Zhang et al., 2020)
MNPs	Immobilized porcine pancreatic lipase	Cross-linking	(Lakshminarayanan et al., 2021)
GO	Immobilized porcine pancreatic lipase	Surface modification	(Pinto et al., 2021)
ZIF-90	PPL@ZIF-90	Adsorption	(Taghizadeh et al., 2021)
CA–CS–CNTs	CA–CS–CNTs-PPL	Surface modification	(Ji et al., 2021)
ILs and MPDA	PPL-ILs-MPDA and PPL-MPDA	Physical adsorption	(Suo et al., 2021)
ACT and FCT	PPL immobilized by ACT and FCT	Physical Adsorption, Covalent Bonding	(Gonçalves et al., 2021)
ACHT	Immobilized lipase	Adsorption	(Gonçalves et al., 2021)
PILs	$PPL_{0.2}$@PIL-COOH-S	Covalent Binding	(Wang et al., 2022)
DUT-5/PVDF	PPL@GOx@DUT-5/PVDF	Covalent Binding	(Hu et al., 2022)
IL-Fe_3O_4@UiO-66-NH_2	PPL-IL-Fe_3O_4@UiO-66-NH_2	Surface modification	(Li et al., 2022)
pSC_4-AuNPs@ZIF-8	PPL@ZIF-8, ZIF-8@PPL pSC_4-AuNPs@ZIF-8@PPL	Encapsulation Surface modification	(Hu et al., 2022)
Fe_3O_4-COOH@UiO-66-NH_2	Fe_3O_4-COOH@UiO-66-NH_2@PPL	Covalent Binding	(Xu et al., 2022)
ILs/MZIF-90	PPL-ILs/MZIF-90	Surface modification	(Suo et al., 2023)
MCM-41 and CTS-MCM-41	immobilized lipase	cross-link	(Zhou et al., 2023)
TpBD-3COOH	PPL@COF	Covalent Binding	(Liu et al., 2023)
acrylamide PPL	Immobilized PPL Zn-imPPL	Covalent Binding	(Jiang et al., 2023)

Fe_3O_4@HM-UiO-66-NH_2	PPL/Fe_3O_4@HMUi-O-66-NH_2	Adsorption	(Yan et al., 2023)
ILs MCaBDC	PPL-ILs/MCaBDC	Covalent Binding	(Suo et al., 2023)
DUT-5	PPL@DUT-5	Adsorption	(Zhou et al., 2023)
NC	immobilized lipase	Covalent Binding	(Iyer, and Chattopadhyay, 2023)

ACT: activated carbon **APTES:** aminopropyltriethoxysilane **CA–CS–CNTs:** functionalized carbon nanotubes **Chi:** chitosan **COF:** covalent organic framework **CS-Fe_3O_4:** magnetic chitosan nanoparticles **DUT-5:** Dresden Dresden University of Technology-5 (Al-MOF) **FCT:** glutaraldehyde-activated carbon **Fe_3O_4-COOH@UiO-66-NH_2:** core–shell metal–organic frameworks composite **Fe_3O_4@HMUiO-66-NH_2:** magnetic core–shell hierarchical mesoporous metal–organic framework **GO:** graphene oxide **IL:** ionic liquid **IL-CS-Fe_3O_4:** functional ionic liquid modified magnetic chitosan nanoparticles **IL-MCMC:** ionic liquids-modified magnetic carboxymethyl cellulose nanoparticles **MCaBDC:** magnetic Ca^{2+}-based metal–organic framework **MCS:** modified magnetic chitosan **mDE:** magnetic diatomaceous earth **MSA:** magnetic alginate nanoparticles **MOF:** metal organic framework **MPDA:** magnetic polydopamine nanoparticles **MZIF-90:** magnetic zeolitic imidazolate framework-90 **NC:** nanocomposite **p(HEMA-GMA):** poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) **PILs:** poly(ionic liquid)s **PPL:** *porcine pancreatic* lipase **pSC_4-AuNPs@ZIF-8:** supramolecular-modified gold nanoparticle complexes **PSS:** polystyrene sulfonate **TpBD-3COOH:** carboxyl functionalized covalent organic framework **ZIF-90:** Zeolitic imidazolate framework

The table includes the supplements used in enzyme immobilization in the researches, the names of the immobilized enzymes, the groups and information of the researchers.

It is thought that the current table will shed light on future research on the enzyme immobilization of *Porcine pancreatic* lipase.

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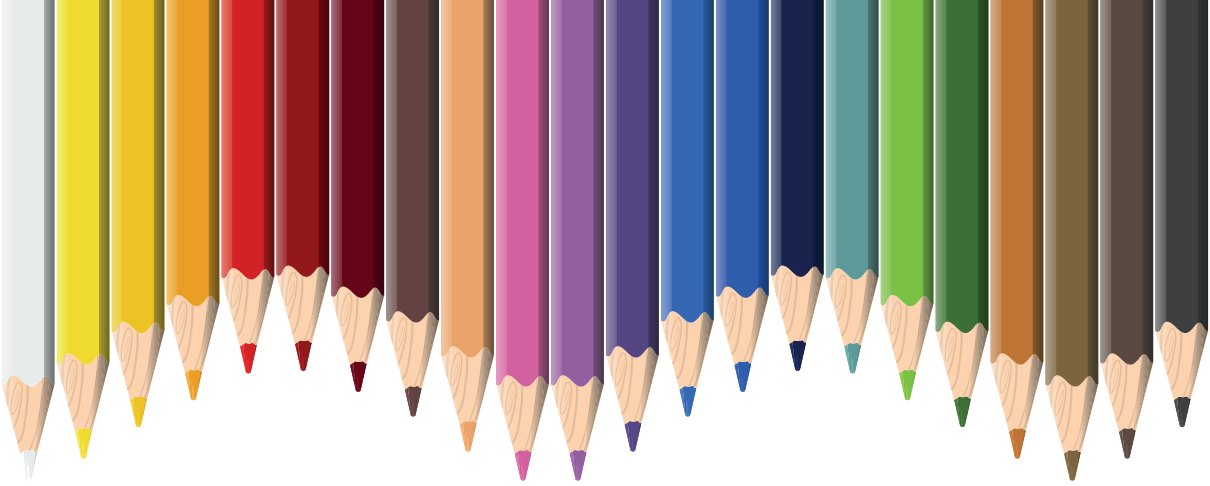

Chapter 6

MICROBIAL SYNTHESIS OF NANOMATERIALS

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1. INTRODUCTION

Materials that possess with one or more structural dimensions ranging from 1 to 100 nanometers (in the nanoscale range) are called nanomaterials (NMs) [1]. NMs exhibit unique properties that enable effective performance and are applied in diverse fields as medicine, energy, cosmetic, food and environmental science [2, 3, 4, 5, 6].

Physical and chemical synthesis methods used to obtain nanoparticles may require high voltage, high temperature, expensive reagents, high-tech apparatus and toxic solvents, and these are factors that increase environmental and human safety and health concerns [7].

There are various methods of producing nano-sized materials which are categorized under two main approaches: (1) bottom up (chemical methods such as sol-gel, co-precipitation, redox, pyrolysis, microemulsion, microwave, photochemical, sonochemical, electrochemical and hydrothermal methods) and (2) top-down (physical methods such as sonication, spray pyrolysis, arc discharge, electro-deposition, evaporation–condensation, vapor and gas phase, ball milling and pulsed laser ablation lithography) approaches (Figure 1) [8].

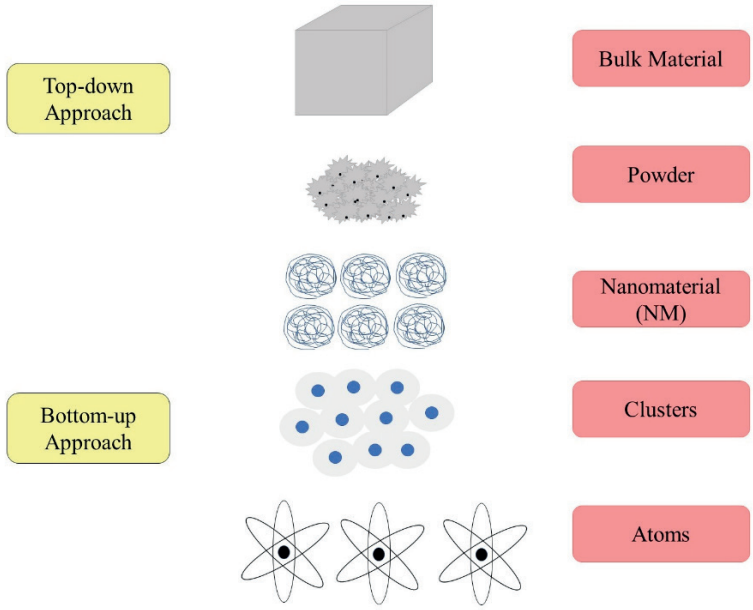

Figure 1. Methods of synthesizing nano-sized materials. [9]

The biological nanoparticle synthesis method which uses biological mediators like biomolecules, biopolymers, bacteria, yeasts, fungi, viruses and plants is considered as a bottom-up approach [10]. Recently, the use of

green synthesis methods in obtaining and developing NMs has increased as an alternative way in the face of the harms caused by the use of traditional approaches, especially considering environmental sustainability concerns. [11]. There are several types of NMs that have been synthesized by biological routes like silver [12], gold [13], copper [14], selenium [15] and ZnO [16].

NM production by green synthesis aims to produce NMs by reducing the damage caused by pollutants known to be used in other processes and indirectly by reducing environmental problems. Therefore, green synthesis of NMs is environmentally friendly and safe compared to chemical/physical synthesis methods, as it produces biocompatible products without the use of toxic chemicals by using biological active components throughout the synthesis process. Additionally, biological components are cheaper than chemical components [17, 18].

Biogenic NM synthesis appears as an advantageous alternative as it is an environmentally friendly and cost-effective method [19]. The term "biogenic" covers a series of methodologies that are utilized ed for NP synthesis by biological mediators (plant, microorganism etc.), owing to their natural NP production ability at the stage of reduction of metal ions to NPs [20, 21].

Due to their easy cultivation, fast growth rate and growth capacity under atmospheric pH, temperature and pressure conditions, microorganisms such as bacteria, fungi, yeast and algae are frequently preferred for NM synthesis. In the synthesis phase, metal ions first adhere to the cell surface which is followed by the reduction of metal ions to NMs by the presence of enzymes synthesized by microorganisms [19]. The biological approach of NM synthesis process is summarized in Figure 2.

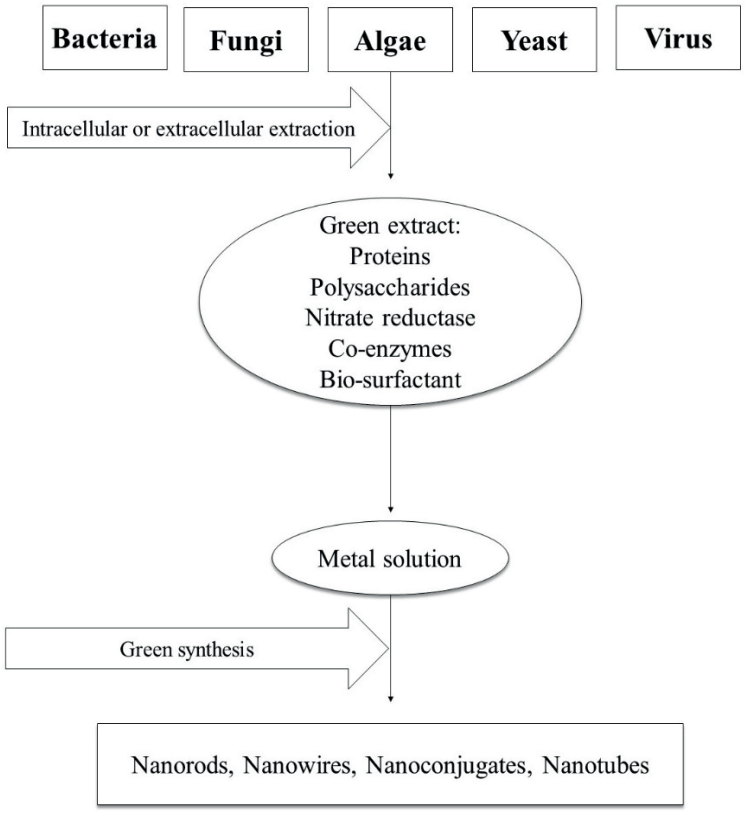

Figure 2. The biological approach to NM synthesis. [22].

2. MICROBIAL NM SYNTHESIS

In biological nanoparticle synthesis, which is considered as environmentally friendly and accepted to be green synthesis, microorganisms are considered as potential biofactories and have recently attracted great attention due to their advantages and technological importance [23]. Various species of bacteria, fungi, yeasts and algae that have the ability to grow under relatively variable conditions have been reported to synthesize microbial NMs with different size, shape and physicochemical features [24, 25].

Biological solutions behave differently when reacted with different metal solutions to form nanoparticles. First, metal ions are retained on the cell surface, then a reduction reaction occurs in the presence of enzymes synthesized by microorganisms and the metal ions are reduced to nanoparticles [19].

The possible microbial NP synthesis process is summarized in Figure 3.

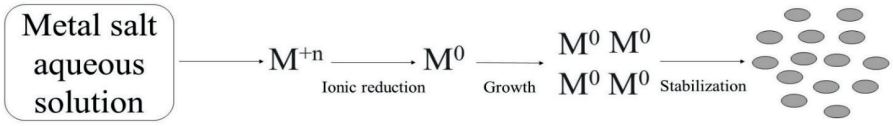

Figure 3. Mechanistic aspect to microbial NM synthesis [19, 23].

2.1. **Bacteria-Mediated NM Synthesis**

Bacteria which are widely used in NM synthesis due to their easy growth conditions, easy purification and high yield are known as the "factory of nanomaterials". They can synthesize NMs either as extracellular or intracellular in their growth media during an incubation period [26]. Extracellular synthesis of NMs is found to be more advantageous than the intracellular method in that it does not require any downstream processes for the collection of synthesized nanoparticles (NPs) and requires less time [27]. The first study about the silver nanoparticle synthesis using bacteria, *Pseudomonas* stutzeri AG259, was reported in 1999 [28].

Extracellular nanoparticle synthesis can be accomplished using biomass, bacterial culture supernatant, or cell-free bacterial extract [29]. Some bacterial species, such as *Lactobacillus* sp. [30], *Aeromonas* sp. [31] and *Vibrio alginolyticus* [32], may have both extracellular and intracellular nanoparticle synthesis ability. Bacteria with this ability not only accumulate NPs within the cells, but also secrete nanoparticles into the external environment [29].

It is thought that the reduction of metallic ions to nanoparticles occurs depending on various factors. The most notable of these are organic functional molecules found in the cell wall that trigger biomineralization. Environmental conditions such as pH, composition of the medium, metallic salt concentration and temperature are among other important factors [33]. The characteristic properties of the nanoparticle, its size, morphology and composition, may vary depending on these environmental parameters [34]. For this reason, optimization studies before biosynthesis are gaining importance.

2.2. **Fungi-Mediated NM Synthesis**

The use of fungal strains in nanoparticle synthesis is called myconanotechnology (MNT). Well-known fugal strains like *Fusarium*, *Aspergillus*, *Verticillium*, and *Pencillium* have all been investigated as potential sources for the production of nanoparticles. Numerous fungal species can be involved in the formation of NMs both intracellularly and extracellularly [35]. When compared to bacteria, fungi have higher nanoparticle production capacity and high tolerance to metals, taking into account the high binding

capacity of metal ions to the cell wall of the biomass [27]. Similar to bacteria, fungi-mediated NM synthesis can be intra or extracellular. In extracellular biosynthesis, fungal extracts play the main role [36]. On the other hand, in intracellular synthesis, micelles convert metal salts into a less toxic form that can be utilized by fungi [37]. Yeast have also been investigated for their NM synthesis capacity. Yeasts have the ability to absorb and accumulate high concentrations of toxic metal ions from their environment [38] and also can adapt to conditions of metal toxicity by using various detoxification mechanisms such as bioprecipitation, chelation or intracellular sequestration [22].

Fungal-based NP synthesis emerges as one of the synthesis routes adopted by researchers due to its widespread application in agriculture. Fungal nanomolecules are used for soil management and applied as nanofertilizers, nanofungicides, nano-insecticides and pesticides [39] . It was also reported that NPs synthesized from fungi, such as *Aspergillus tubingiensis*, *Fusarium verticillioides*, *Alternaria* sp., *Penicillium aculeatum*, *Trichophyton* sp., etc have antibacterial and antifungal activities against *Streptococcus pneumonia*, *P. aeruginosa*, and *K. pneumoniae*, multidrug resistant bacteria like *S. aureus*, *Shewanella putrefaciens* and yeast *C. albicans* [40-43] .

2.3. Algae-Mediated NM Synthesis

Algae stand out as a rich source of biomolecules in terms of carbohydrates, fats, nucleic acids, pigments, proteins and secondary metabolites that they have. Algal species such as *Bifurcaria bifurcate*, *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, *Ecklonia cava*, *Fucus vesiculosus*, *Oscillatoria willei*, *Pithophora oedogonia*, *Sargassum muticum*, *Sargassum wightii*, *Spirulina platensis*, S*toechospermum marginatum* have been reported to fabricate metal and metal oxide nanoparticles [44].

It is possible to reduce the metal ion to a zero-valent state when biochemical compounds such as carbohydrates, chlorophyll, fats, minerals, oils, phycobilin, pigments, polyunsaturated fatty acids, proteins, vitamins and other phytochemicals found in algae act as reducing and capping agents [45].

There are various reports about producing intracellular or extracellular metallic NP using algae like *Gelidium amansi* [46], *Neochloris oleobundans* [47], *Chlorella pyrenoidosa* [48] for silver NP; *Galdieria* sp. for zinc and iron NPs [49]; Dunaliella *tertiolecta*, *Tetraselmis suecica* and *Chlorella kessleri* for copper NPs [50].

According to the accepted mechanistic approaches, the metal ions are trapped on the surface of the cells and reduced in the presence of enzymes in the extracellular synthesis process of NPs. In intracellular synthesis, metal ions are transferred to the cell to transform into NPs in the presence of enzymes [51]. Figure 4 summarizes the types of NP synthesis mechanisms by algae.

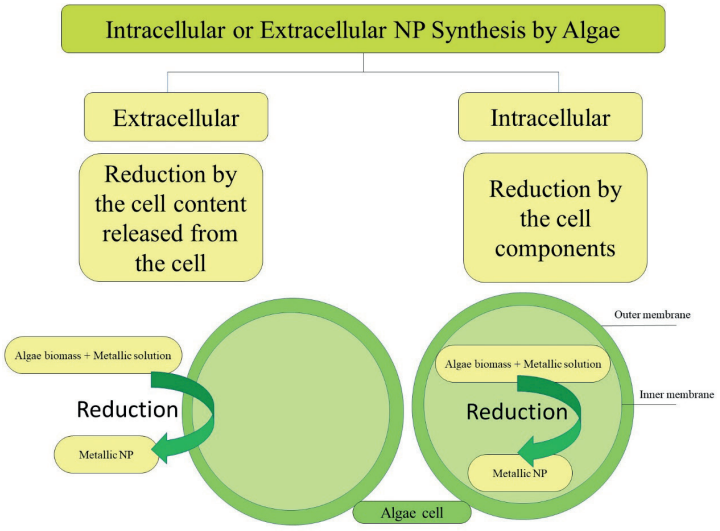

Figure 4. Intracellular and extracellular green synthesis of NP by algal cells [51].

3. ANTIMICROBIAL ACTIVITY OF MICROBIAL NMs

The burden of infectious diseases spread by microorganisms is a serious health problem worldwide, especially in economic terms. Biosynthesized metal NMs have becomed popular wth their potential to combat microbial infections involving multidrug-resistant (MDR) pathogenic microorganisms. When compared to chemical and physical methods, "green synthesis" of metallic nanoparticles is accepted as harmless due to their non-toxic synthesis procedure [52].Although the exact mechanism of the antimicrobial activity of nanoparticles against microorganisms is still being investigated, some possible factors stand out.

Possible antibacterial mechanisms of biosynthesized metal nanomaterials are listed as [52]:

- Inhition of DNA replication,
- Protein leakage,
- Protein damage,

- Mithocondrial dysfunctiononing,
- Physical attachment of NPs with cell,
- Disruption of cell membrane/cell wall,
- Direct entrance of NPs,
- Interruption of electron transportation.

Due to their fast production and strong activity against bacteria, silver nanoparticles (AgNPs) are mostly reserched NPs among other metallic NPs [53-56].

4. APPLICATIONS of NMs

Biogenic NPs have several area of use in chemical, physical and biological processes such as health, food, environment, agriculture and industry (Figure 5). They can be used for biosensors, bioimaging, biomedical equipments, drug optimization and delivery in biomedical industry. Besides, they can be involved in producing daily use products such as toothpastes, deodorants and cosmetics. Nowadays, NPs can be incorporated in active food packaging systems to prolong the shelf life of food. Green-sythesized NMs find their place in environmental solution systems such as energy storage or solar solutions [57].

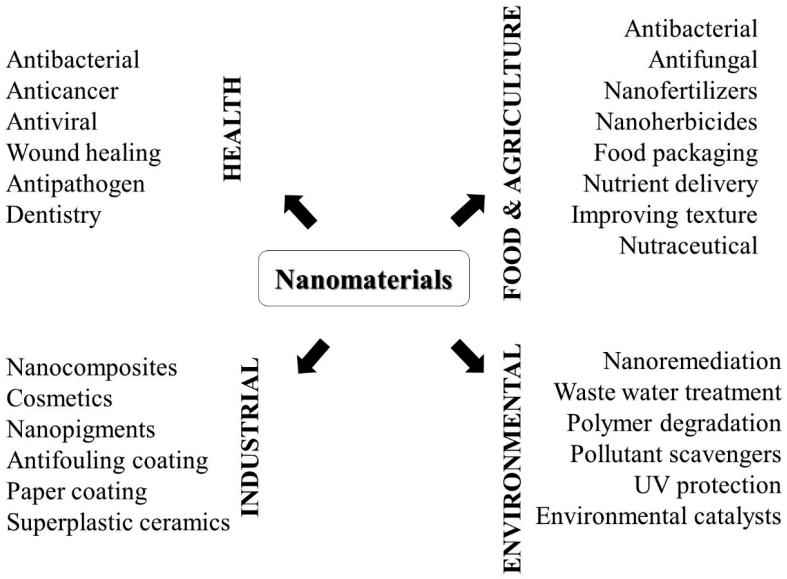

Figure 5. Use of NMs in different industrial areas [57]

5. CONCLUSION

Biosynthesis of NPs is an ecofriendly route for obtaining nano-sized materials with high potential. Those nanomaterials are investigated for their alternative use in several industries. It has been proved that biogenic NPs exhibit strong biological features as antimicrobial, anticancer and antibiofilm activites. Microorganisms which are called as nanofactories have been gaining much importance by researchers with their less requirements that are necessary for nanosynthesis approaches. Future studies will reveal out the exact nanobiosynthesis mechanisms of microorganisms and their superiority over the chemical or physical synthesis protocols.

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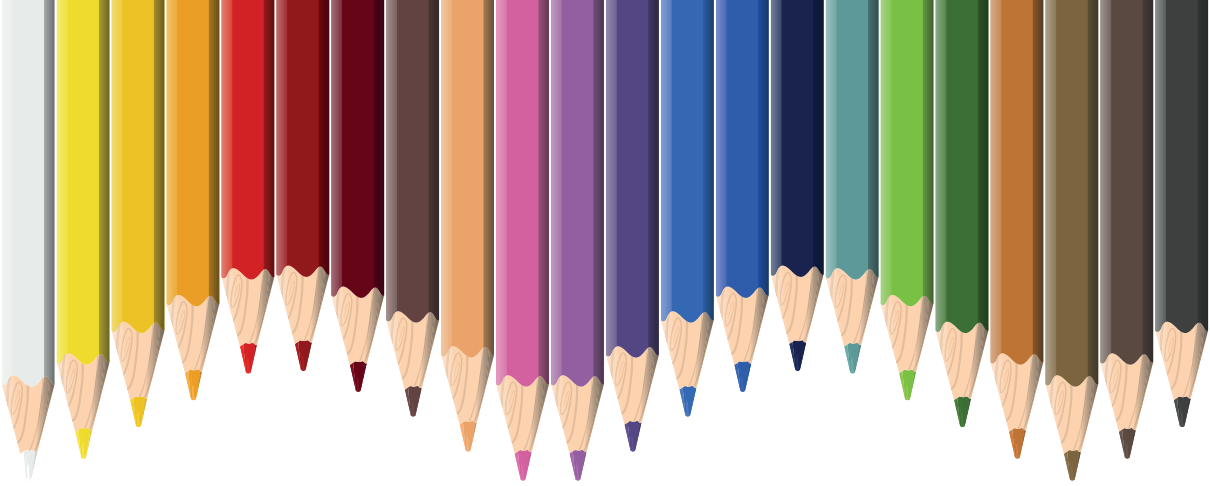

Chapter 7

EFFECTS OF ROADKILL ON BREEDING OF SOME AMPHIBIAN SPECIES IN TÜRKİYE

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Introduction

Declines of Amphibians around the world and the potential causes are the subject of current studies (Blaustein & Wake, 1990; Wyman, 1990; Blaustein et al., 1994). Varied factors cause the declines of amphibians. Examples of these factors contain climate change, predators and introduction of exotic species, habitat destruction, UV rays, pollution and pesticides, natural population variability, and human consumption (Blaustein et al., 1994). Many declines are due to human factors (Hels & Buchwald, 2001). One of the serious factors affecting the decline of amphibians is road mortality. Nevertheless, few studies have focused on this problem (Fahrig et al., 1995; Hels & Buchwald, 2001). Fahrig et al. (1995) noted that density and volume of traffic have been greatly increased in recent years. Therefore, it can be assumed that as traffic increases, deaths and injuries caused by vehicles also increase.

Networks of the roads supplies rapid and comfortable transportation of people and goods. However, so many negative effects emerge on natural habitats (Forman et al., 1998; Trombulak & Frissell, 2000; Forman et al., 2003; Fahrig & Rytwinski, 2009). The traffic and roads have direct (mortality or injury which happen pending road construction and because of collision with vehicles) and indirect (degradation and loss of habitat, microclimatic changes, chemical pollution, increasing in the level of noise, and light) influences on animals (Fahrig & Rytwinski, 2009; van der Ree et al., 2015; Lengagne, 2008; Buchanan, 1993), and (Andrews et al., 2006). These influences may cause a decline in density and size of the populations (Fahrig et al., 1995; Hels & Buchwald, 2001), a decrease in genetic diversity (Noël et al., 2007), reproductive output (Jochimsen et al., 2004), reduction of survival rates, and alteration of age and sex ratios (Bonnet et al., 1999; Aresco, 2005), as well as movements of animals e.g. road avoidance or/and barrier effect (Forman, 1998; Trombulak & Frissell, 2000). Each year, millions of animals are exposed to roadkill (Andelković & Bogdanović, 2022).

Grade of protection impresses the risk of exposing roadkill. Even the protected areas may have a high percentage of roadkills (Garriaga et al., 2012). Furthermore, areas with no roads have the highest conservation capacity (Crist et al., 2005; Chen & Roberts, 2008; Selva et al., 2011; Ibisch et al., 2016). Determinants dependent on composition and structure of landscape define the existence of animals on roads and, that's why, the possibility of exposed to roadkill by a vehicle (Santos et al., 2008; Santos et al., 2013; Sillero, 2008; Ha & Shilling; 2018). At the same time, some elements are determined according to road characteristics (e. g. number of vehicles, type of road) and also by driver behavior (Gunther et al.,1998; Hels & Buchwald, 2001; Clevenger et al., 2003; Saeki & Macdonald, 2004; Santos et al., 2008; Matos et al., 2012). For instance, some of the researches showed that roadkill of the snakes are intentional (Langley et al., 1989; Paul Ashley et al., 2007; Secco et al., 2014).

For small-bodied animals (e.g. small amphibians, mammals, and reptiles), the roads may be especially deadly (Adams and Geis, 1983; Ashley and Robinson, 1996; Fahrig et al., 1995; Hodson, 1966). Amphibians that breed in pond, that migrate in large quantities to and from breeding areas, are especially undefended to collisions with vehicles (Fahrig et al., 1995; Hels and Buchwald, 2001; Palis, 1994). The vehicle crashing can be related to particular amphibian behaviors of (e.g., escaping, internalizing threat displays, staying immobile) in reply to intensive light or sound related to vehicles. Distinctions in these reactions across species may find out which species are especially vulnerable to roadkill.

Roadkills were quantified in varied animal taxa such as toads (van Gelder, 1973; Cooke, 1995), amphibians, birds, reptiles, and mammals (Hansen, 1982; Fuellhaas et al., 1989), butterflies (Munguira and Thomas, 1992), snakes (Rosen and Lowe, 1994), birds, reptiles, and mammals (Drews, 1995), deer and other ungulates (Romin and Bissonette, 1996; Groot Bruinderink and Hazebroek, 1996) in several studies. Because of their activity pattern, preferred habitats, and population structure, aquatic amphibians are excessively vulnerable to roadkill when compared to other species.

Moreover, amphibians are the most often road-killed taxa having decrement rates of 60-90% in researches conducted on vertebrates (Fahrig et al., 1995; Glista et al., 2008). In the world, amphibian species are endangered and suffered from various threats such as pollution, habitat fragmentation and modification, diseases, invasive species or climate change (Beebee, 2013; Rytwinski & Fahrig, 2015; Gibbons et al., 2000; Andrews et al., 2015). Fatal collisions with vehicles are the most direct negative effect of road traffic on animal populations (Rytwinski & Fahrig, 2015). Roadkill affect all taxonomic groups in different ways. Amphibians (salamanders, newts, and toads) are mostly affected by roadkill when they cross roads along migration between their breeding and hibernation sites (Andrews et al., 2015; Forman et al., 2003; Glista et al., 2008; D'Amico et al.,2015; Teixeira et al., 2013).

The breeding season is the critical period for amphibians when the number of individuals exposed to roadkills increases rapidly (Orłowski et al., 2008). For amphibians that migrate to their areas of breeding, road mortality recorded at grades changing from 19% (Gibbs & Shriver, 2005) to 98% (Hels & Buchwald, 2001). The highest peaks are generally recorded during spring (the starting season for breeding migration) under temperate zone circumstances.

In the present study we tried to examine how roadkills could endanger the survival of amphibians by presenting some cases where both anuran and urodela species in Türkiye were exposed to roadkills during their breeding seasons.

Result and Discussion

In the field studies carried out in Kuşçu village of Maçka district of Trabzon province in 22 May 2019, it was observed that the female individual of the Common Toad, *Bufo bufo* (Linnaeus, 1758) was crushed to death on the road during the amplexus behavior, while the male individual was not damaged and continued its amplexus behavior (Figure 1A). Next year on the same road, another couple of *B. bufo* were found that both of the individuals (male and female) were crushed to death on the road during amplexus 11 June 2020 (Figure 1B).

During the spring, summer, and autumn seasons of 2019 and 2020, our observations were made in seventy-five localities on Atasu-Kuşçu highway (TR D885-01) and sixty-four localities on Pazar-Hemşin highway (TR D010/53-04) which were located in Trabzon and Rize provinces, respectively in the eastern Black Sea region of Türkiye. The lengths of observed roads were 12 km in Atasu-Kuşçu highway and 10 km in Pazar-Hemşin highway.

In addition, we performed observations in twent localities on a stabilized road (TR D01/61.28) located in Arpalı village of Köprübaşı district, Trabzon province during the summer and autumn seasons of 2019. The length of observed roads was about 5-6 km.

Figure 1. *The carcasses of females and males of Bufo bufo exposed to roadkill when they perform amplexus behavior on the Atasu-Kuşçu highway in 2019* **(A)** *and 2020* **(B)**.

Many protection precautions were made to reduce roadkills Grilo et al., 2009; Ascensão et al., 2013; Bager & Fontoura, 2013). Because resources are restricted, mitigation precautions must be conducted at least in places where higher roadkill rates are occurred, called hotspots (Matos et al., 2012; Sillero, 2008). Hotspots may be defined with spatial statistics and procedures of modelling (Malo et al., 2004), ecological niche factor analysis, binary logistic regression, nearest-neighbour hierarchical clustering (Gomes et al., 2009), Ripley's K function (Clevenger et al., 2003; Ramp et al., 2005; Teixeira et al., 2013) or Getis–Ord Gi* (Shilling & Waetjen, 2015). Hotspots are attached to high roadkill rates, points of animal crossings, barriers for crossing roads safely (where the animal is blocked in the road), and (Matos et al., 2012).

Our findings indicating that these toads exposed to roadkills during two consecutive breeding periods while they perform amplexus activity on the same route clearly shows that there are hotpoints on this route. Reports on abiotic (topography of roadside) and biotic (vegetation) data linked to mortality of toads are limited (Clevenger et al., 2003). This viewpoint is relevant, because toads use the same pathways for migratory from hibernation to breeding areas every year. Sites having migratory routes which present potentially high roadkill should be defined and effective cautions should be adopted to decrease roadkills.

The individuals of *Bufo bufo* have been frequently killed on roads (Santos et al., 2007; Brzeziński et al., 2012; Matos et al., 2012). Roadkills usually associated with migration to breeding areas (Feldmann & Geiger, 1989; Orlowski, 2007; Van Gelder, 1973), when hundreds of individuals are exposed to roadkill along the breeding period (Cooke, 1995; Frétey et al., 2004). Depending on weather conditions, breeding migrations of *Bufo bufo* generally last between 10 days and 1 month during the beginning of the breeding season (Gittins 1983; Juszczyk 1987).

Moreover, we observed that many adult male and female individuals were exposed to roadkill on Atasu-Kuşçu highway in April and May of both 2019 and 2020, which are the beginning months of the breeding period of these toads. Reproduction occurs in this species from March-June, usually late April-May (https://amphibiaweb.org). On the other hand, Koç-Gür et al. (2020) reported a high number of road-killed individuals of *B. bufo* in Pazar-Hemşin and Hemşin-Gito highways and suggested that returning from their long breeding migration routes may increase exposure to road deaths in October. These toads usually hibernate in groups or singly from September (or beginning of November) to March-June, according to the altitude and latitude. Usually, hibernation occurs on land and occasionally in streams and springs. In general, the hibernation ends in April-May (https://amphibiaweb.org).

Bufo bufo has been classified as LC (Least Concern) in the IUCN Red List of Threatened Animals since 2009 (Agasyan et al., 2009). Although *B. bufo* is listed in the LC category, like other amphibian species, the number of individuals of this species is gradually decreasing due to the negative effects of human activities, especially during the breeding period of these toads. It is essential that the necessary precautions be taken by the state authorities to prevent these toads from being subjected to road kill on the Atasu-Kuşçu highway in the months of April and May.

Our surveys performed in 2019 (on the Pazar-Hemşin highway located in Rize province) revealed that the number of dead individuals of the Iranian

long-legged frog, *Rana macrocnemis* (Boulenger, 1885) exposed to roadkill was higher in May (the beginning of the breeding season in this area) than in other months. In this highway, a road-killed individual of *R. macronmemis* was photographed on 19 September 2019, (Figure 2).

Figure 2. *A dead female of Rana macrocnemis exposed to roadkill on Pazar-Hemşin highway.*

During our field studies which were made on the Pazar-Hemşin highway located in Rize province in 2020, we observed that the number of dead individuals of the Oriental tree frog, *Hyla orientalis* (Bedriaga, 1890) exposed to roadkill was higher in April and May, which is the beginning of the breeding season, than in other months. After these two months, we encountered the highest number of road deaths in September, when frogs leave their aquatic environments, which are their breeding grounds, and begin reverse migration for hibernation. Figure 3 shows a dead individual of *H. orientalis* exposed to roadkill on 25 May 2020.

In the different roads of Anatolia, Tok et al. (2011), in May and June months of 2015-2019, and Koç-Gür et al. (2020), in May and October months of 2018, notified roadkill cases of *Rana macrocnemis*.

Figure 3. *A carcass of Hyla orientalis found on the Pazar-Hemşin highway.*

Similar to our findings, Anđelković and Bogdanović (2022) reported that the number of killed individuals of *Hyla arborea* increased in the beginning of the season and it reached a peak in October on a road section of 4.2 km located on the edge of Special Nature Reserve "Obedska bara" in Serbia.

Moreover, during our field observations in 2020 on a stabilized road in Arpalı village of Köprübaşı district of Trabzon province, we noticed that the number of dead individuals of the Caucasian salamander, *Mertensiella caucasica* (Waga, 1876) exposed to roadkill was higher in June (the beginning of the breeding activities of these salamanders in this area) than in other months. Figure 4 shows a road-killed individual of *M. caucasica* exposed to roadkill on July 8, 2020, in the study area.

Salamanders can also be subject to roadkill, although not to the same extent as toads. D'Amico (2009) stated that the Iberian ribbed newt, *Pleurodeles waltl* (Michahelles, 1830) was in the third ranking of road-killed species in his study due to massive migratory behavior of the species.

Figure 4. *A female individual of Mertensiella caucasica exposed to roadkill on a stabilized road in Arpalı village of Köprübaşı.*

In conclusion, results of our study clearly show that roadkill negatively affects the breeding of the amphibian species living in the study areas. In particular, the presence of many adult male and female individuals *Bufo bufo*, especially on the Atasu-Kuşçu highway, who are exposed to roadkill and cannot reproduce, may endanger the survival of the species in this area in the future.

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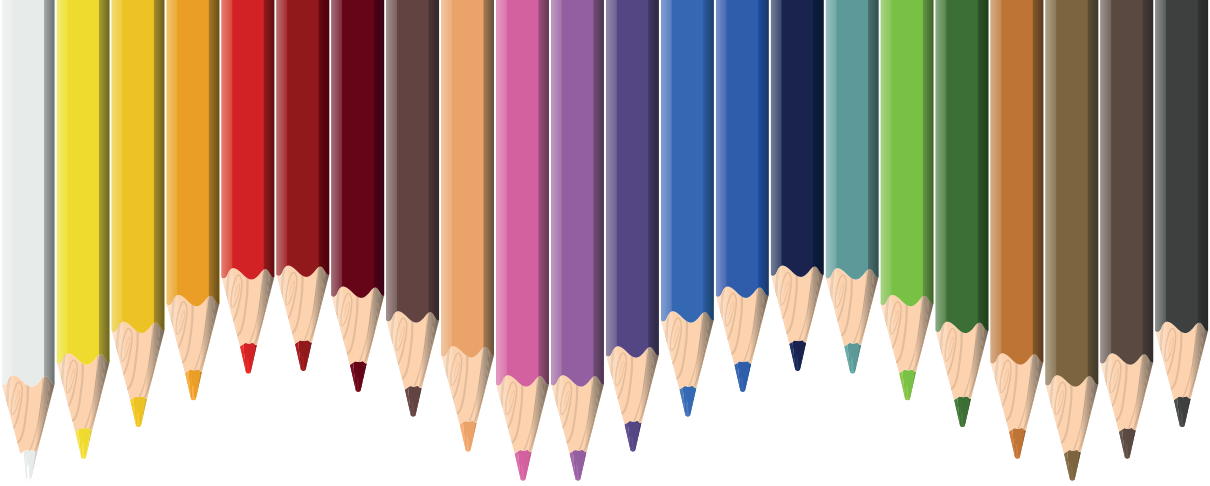

Chapter 8

A REVIEW ON THE BIOLOGICAL ACTIVITIES OF URTICA DIOICA

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Introduction

Plants are very important natural products. It is used by people for many purposes such as food, medicine, and spice (Mohammed et al., 2020a; Korkmaz et al., 2021). Due to their nutritional properties, plants have been important for people in their daily lives (Mohammed et al., 2022). The important nutritional elements such as vitamins and minerals they contain meet people's needs (Mohammed et al., 2020b). In addition to these features, many plant species are also very important from a medicinal point of view (Sevindik et al., 2017). Many studies in the literature have reported that it has many properties such as anticancer, antidiabetic, antimicrobial, antioxidant, anti-inflammatory, and DNA protective (Mohammed et al., 2018; Mohammed et al., 2019; Mohammed et al., 2021b; Comlekcioglu et al., 2022; Unal et al., 2022; Kalkan et al., 2023; Uysal et al., 2023). Plants are natural products widely used in traditional medicine. Thanks to the many compounds they contain, their biological activities are quite high. In this context, investigating the biological activities of plants is very important in terms of their usage potential (Pehlivan et al., 2018; Mohammed et al., 2023a). In this study, the biological activities of *Urtica dioica* reported in the literature were compiled.

Urtica dioica (Urticaceae) is known as nettle, burnt nettle, nettle leaf, or simply nettle or sting. Nettle, a perennial herbaceous flowering plant, is quite common around the world. It has been widely used by humans since ancient times as a source of traditional medicine, food, tea and textile raw materials (Lukešová et al., 2017).

Biological activity

Plants exhibit many biological activities thanks to the secondary metabolites they produce (Sevindik et al., 2023). In this study, in vitro and in vivo biological activity studies of Urtica dioica reported in the literature were compiled. As a result of the research, it was seen that extracts of the plant such as ethyl acetate, hydroalcoholic, petroleum ether, n-butanol, ethanol, aqueous, methanol, water and hexane were used. Biological activity studies of *U. dioica* reported in the literature are shown in table 1.

Table 1. Biological activities of Urtica dioica

Biological activity	Solvents	Geographic regions	References
Antioxidant, antimicrobial, apoptotic, hepatoprotective, antihelmintic, antimutagenic, cytotoxic, anticancer, antinociceptive, anti-inflammatory	Ethyl acetate, hydroalcoholic, petroleum ether, n-butanol, ethanol, aqueous, methanol, water, hexane	Iraq, India, Iran, Spain, Turkey, Italy, Tunusia, Malaysia	(Majd et al., 2003; Özçelik et al., 2009; Güder and Korkmaz, 2012; Khare et al., 2012; Saklani and Chandra, 2012; Sarma Kataki et al., 2012; Modarresi-Chahardehi et al., 2012; Fattahi et al., 2013; Ghaima et al., 2013; Hajhashemi and Klooshani, 2013; Di Sotto et al., 2015; Joshi et al., 2015; Ghasemi et al., 2016; Maaroufi et al., 2017; Mohammed et al., 2021; Flórez et al., 2022; Karakol et al., 2022)

Antioxidant activity

Free radicals are oxidant compounds produced as a result of routine metabolic activities (Krupodorova and Sevindik, 2020). While these oxidant compounds are not harmful at low levels, they can cause cellular damage as their levels increase. When the levels of oxidant compounds increase, the antioxidant defense system comes into play and suppresses oxidant compounds (Bal et al., 2019; Gürgen and Sevindik, 2022). However, in some cases, the antioxidant defense system is inadequate. In such cases, oxidative stress occurs. As a result of oxidative stress, serious diseases such as multiple sclerosis, cancer, and neurodegenerative diseases may occur (Selamoglu et al., 2020; Saridogan et al., 2021; Bal et al., 2023). Supplementary antioxidants can be used to reduce the effect of oxidative stress (Eraslan et al., 2021). Plants are natural products with high potential to be antioxidant supplements (Akgül et al., 2022). In this study, antioxidant activity studies of U. dioica reported in the literature were compiled. In a study conducted in Iraq, the antioxidant status of ethyl acetate extract of U. dioica was investigated. As a result of the study, it was reported that nettle caused 76% lipid peroxidation in linoleic acid inhibition by thiocyanate method (FTC) (Ghaima et al., 2013). Ferulic acid, a potential phenolic antioxidant found using the DPPH free radical scavenging activity of the hydroalcoholic extract of U. dioica produced in India, was examined through HPTLC. As a result of the research, it was reported that *U. dioica* hydro-alcoholic extract showed positive results in terms of antioxidant activity with an LC50 value of 88.33 µg/mL (Khare et al., 2012). In another study conducted in India, the antioxidant properties of petroleum ether, ethyl acetate, n-butanol and ethanol extracts of *U. dioica* were evaluated. As a result of the study, the LC50 values of different solvent extracts (Petroleum ether, ethyl acetate, n-butanol, ethanol) of U. dioica in the DPPH model were 215.96

µg/mL, 78.99 µg/mL, 168.24 µg/mL and 302.90 µg/mL, respectively. It has been reported that the LC50 value of L-Ascorbic acid is 26.24 µg/mL, and the LC50 values for nitric oxide radical scavenging activity are 172.38 µg/mL, 101.39 µg/mL, 141.23 µg/mL, 202.26 µg/mL and 55.38 µg/mL (Joshi et al., 2015). In a study conducted in Iran, it was reported that the aqueous extract of *U. dioica* has antioxidant potential (Fattahi et al., 2013). In a study conducted in Spain, DPPH and ABTS tests and antioxidant status of methanol, water and ethanol extract of *U. dioica* were analyzed. As a result of the study, it was reported that the most effective method, DPPH, and the best results were obtained in water extraction of nettle extracts (91.08%) (Flórez et al., 2022). In a different study in India, the antioxidant status of methanol extract of *U. dioica* was investigated. As a result of the study, it was reported that the total antioxidant activity determination system in linoleic acid showed higher antioxidant activity than the extract at 250 µg/mL concentration and tocopherol at 250 µg/mL concentration. It was also reported that the inhibition percentage of peroxidation of the extract in the linoleic acid system was 62.34%, the inhibition percentage of tocopherol at 250 g/mL concentration was 34.41%, the percentage of DPPH scavenging effect was 69.05% and the nitric oxide scavenging activity was 63.76-82.02% (Sarma Kataki et al., 2012). . In a study conducted in Turkey, the antioxidant properties of the hydroalcoholic extract of different parts of *U. dioica* were analyzed by total antioxidant activity, reducing power, superoxide anion radical scavenging, hydrogen peroxide scavenging, free radical scavenging and metal chelation activity tests (Güder and Korkmaz, 2012). In a study conducted in Italy, *U. dioica* was reported to have LC50 values of 19.9 µg/mL and 75.3 µg/mL, with a remarkable scavenging activity against ABTS radical and superoxide anion, respectively (Di Sotto et al., 2015). In another study conducted in Turkey, total antioxidant (TAS), total oxidant (TOS) and oxidative stress index (OSI) values of U. dioica ethanol extract were investigated. As a result of the study, it was reported that the TAS value of the plant extract was measured as 7.817, TOS value 10.866 and OSI value 0.139 (Mohammed et al., 2021c). In this context, according to literature data, it has been observed that *U. dioica* has significant antioxidant potential.

Antimicrobial activity

In recent years, difficulties have been experienced in the fight against microorganisms (Baba et al., 2020). The increase in the number of resistant microorganisms, especially due to unconscious use of antibiotics, makes this fight insufficient (Mohammed et al., 2023b). Possible side effects of synthetic drugs have led researchers to discover natural antimicrobial drugs. In this context, plants are an important resource (Bal et al., 2017; Islek et al., 2021). In this research, antimicrobial activity studies of *U. dioica* reported in the literature were compiled. In a study conducted in Iraq, the antimicrobial status of the ethyl acetate extract of U. dioica against *Aeromonas hydrophila,*

Salmonella typhi, Staphylococcus aureus, Bacillus cereus and *Escherichia coli* was investigated. As a result of the study, it was reported that nettle gave a wide inhibition zone to S. typhi (22mm) (Ghaima et al., 2013). In a study conducted in Turkey, the antimicrobial status of the oil obtained from the seed of *U. dioica* against *Escherichia coli*, *Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae, Acinetobacter baumannii, Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis, Candida albicans* and *Candida parapsilosis* was analyzed. As a result of the study, it was reported that the minimum inhibitory concentration (MIC) value range was 16-64 µg/mL against bacteria and 16 µg/mL against fungi (Özçelik et al., 2009). In a study conducted in Iran, the antimicrobial properties of aquatic and ethanolic extracts of *U. dioica* against *Pseudomonas aeruginosa*, *Bacillus subtilis, Staphilococcus aureus, Escherichia coli* and *Candida albicans* were evaluated. As a result of the study, it was reported that the ethanolic extract (80%) of *U. dioica* showed an antimicrobial effect on *Pseudomonas aeruginosa, Bacillus subtilis, Staphilococcus aureus, Escherichia coli* and *Candida albicans* (Majd et al., 2003). In a study conducted in India, it was reported that ethanolic root extracts of *U. dioica* showed 17 mm, 16 mm and 14 mm inhibition zone effects against *Escherichia coli, Streptococcus pyogenes* and *Salmonella entericatyphim* strains that cause food poisoning (Saklani and Chandra, 2012). In a study conducted in Tunisia, it was reported that the water extract of *U. dioica* caused a decrease in the growth rate between two fungi, *Penicelleum notatum* and *Fusarium oxysporum*, between 1 and 2.5 mg/mL (Maaroufi et al., 2017). In a study conducted in Malaysia, it was reported that *Bacillus subtilis* using butanol extract of *U. dioica* was effective against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Vibrio parahaemolyticus* (Modarresi-Chahardehi et al., 2012). In this context, according to literature data, it has been observed that *U. dioica* has significant antimicrobial potential.

Other activity

It has been reported in the literature that *U. dioica* has different biological activities in addition to antioxidant and antimicrobial activities. In a study conducted in Iran, it was reported that the aqueous extract of *U. dioica* had significant antiproliferative activity on the human breast cancer cell line (MCF-7) and fibroblasts isolated from foreskin tissue using the MTT assay (Fattahi et al., 2013). In a study in India, the hepatoprotective and antihelmintic status of the methanol extract of *U. dioica* was investigated. As a result of the study, it was determined that maximum hepatoprotection was observed at the dose of 400 mg/kg, as suggested by the decreasing serum level for the hepatoprotective state, and ALT, AST, ALP and total bilirubin levels went towards normalization. It has also been reported to cause a significant decrease in malonyldehyde (MDA) level and a significant increase in superoxide dismutase (SOD) level. In the same study, it was reported that

there was a dose-dependent increase in the anthelmintic activity of the extract against earthworms (*Pheretima posthuma*) (Sarma Kataki et al., 2012). In a study conducted in Italy, it was reported that *U. dioica* exhibited strong antimutagenic activity against the mutagen 2-aminoanthracene (2AA) in all tested strains (Di Sotto et al., 2015). In another study conducted in Iran, the cytotoxicity of *U. dioica* on MKN45 and HT29 cancer as well as normal human foreskin fibroblast (HFF) cells was investigated. As a result of the study, it was reported that LC50 values of 24.7, 249.9 and 857.5 μg/mL occurred for HT29, MKN45 and HFF cells, respectively, after 72 hours of treatment (Ghasemi et al., 2016). In a study conducted in Turkey, the anticancer effect of *U. dioica* against breast cancer cell line was analyzed. As a result of the study, it was reported that the number of annexin positive cells was higher in cell lines treated with the sample extract than in untreated control cells (Karakol et al., 2022). In another study conducted in Iran, it was reported that the extract for the antinociceptive and anti-inflammatory status of *U. dioica* reduced acetic acid-induced abdominal twitching depending on the dose (Hajhashemi and Klooshani, 2013).

Conclusion

In this study, the biological activities of *U. dioica* reported in the literature were compiled. According to the findings, it has been determined that the plant may be an important source of antioxidant, antimicrobial and anticancer activities. According to literature data, *U. dioica* is thought to be an important natural resource that can be used in pharmacological studies.

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